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14. ABSTRACT

Signal transducer and activator of transcription 5b (STAT5b) is a transcription factor which promotes growth and survival of a number of cancers, including breast cancer. The objective of this work was to elucidate the role of STAT5b in breast cancer cell migration. Through siRNA knockdown technology, the necessity of STAT5b for migration of two highly aggressive, highly migratory, breast cancer cell lines (BT-549 and MDA-MB-231), was explored. Knockdown of STAT5b inhibited migration of both cell lines by 60-80%. More specifically, STAT5b knockdown suppressed migration of these cells to the extracellular matrix component fibronectin, suggesting that STAT5b is an important signaling component in β1 integrin-mediated migration of breast cancer cells. Inhibition of migration upon STAT5b knockdown was rescued by wild-type STAT5b as well as transcriptionally inactive Y699F- and dominant-negative forms of STAT5b. In contrast, an SH2 domain defective R618K-STAT5b did not rescue migration. These data indicate that STAT5b-mediated transcription is not required for promoting migration, but SH2 domain interactions are necessary. Upon spreading, STAT5b knockdown cells adopted a striking phenotype characterized by the formation of multiple, highly contractile protrusions. In accordance with these findings, knockdown cells exhibited high levels of myosin light chain phosphorylation, independent of attachment. Additionally, knockdown of STAT5b correlated with loss of polarity, resulting in lack of directionality during wound closure. In summary, the data presented here identifies a novel, SH2-dependent function of STAT5b in regulating β1 integrin-mediated migration of highly aggressive breast cancer cells.

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Introduction

Signal Transducers and Activators of Transcription (STATs)

The **S**ignal **T**ransducers and **A**ctivators of **T**ranscription (STATs) are a family of transcription factors originally identified as downstream mediators of type I interferon (IFN) signaling (Darnell, 1997). There are seven mammalian STATs: STAT 1, 2, 3, 4, 5a, 5b, and 6. STATs 2, 4, and 6 are activated by only a few distinct cytokines, whereas STATs 1, 3, 5a, and 5b are activated by a diverse range of ligands, including both cytokines and growth factors. STAT5b, the focus of our studies, is activated by interleukins (IL) 2, 3, 5, 7, 9, and 15, growth hormone (GH), prolactin (Prl), epidermal growth factor (EGF), erythropoietin (EPO), thrombopoietin (TPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), and Abl kinase (Bromberg, 2000).

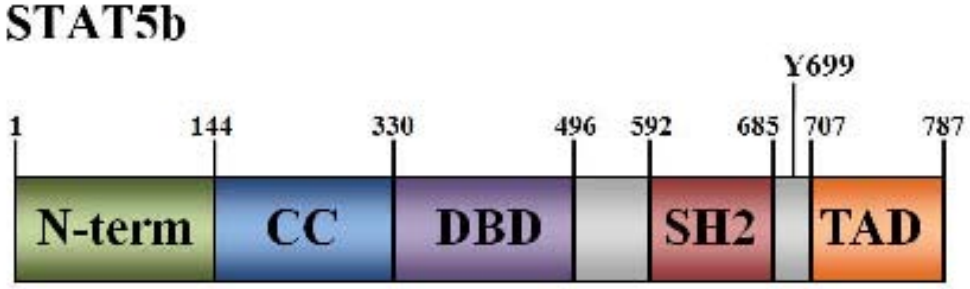
STAT Structure

All members of the STAT family have a conserved domain structure. Each protein consists of an N-terminal oligomerization domain (N-term), a coiled-coil domain (CC), a DNA-binding domain (DBD), a linker region, a Src homology 2 domain (SH2), and a C-terminal transactivation domain (TAD) (Figure 1).

The N-terminus is a hook-like structure of 8 alpha helices. Tryptophan 37 (W37) and lysine 70 (K70) residues in the N-terminus mediate tetramer formation (John et al., 1999). W37 is also important for receptor targeting, as a W37A-STAT4 mutant cannot be phosphorylated by the IFNα receptor (Murphy et al., 2000). Additionally, the N-terminus binds transcriptional coactivators in the nucleus. For example, threonine 92 (T92) of STAT5a is required for binding the coactivator p300/CBP (Gewinner et al., 2004).

Figure 1: STAT5b Domain Structure

STAT proteins are comprised of 6 conserved domains. This figure illustrates the domains of the STAT family member, STAT5b. STAT5b is 787 amino acids in length and contains an N-terminal domain (N-term), a coiled-coil domain (CC), a DNA binding domain (DBD), a linker region, a Src homology 2 domain (SH2), and a C-terminal transactivation domain (TAD). The conserved tyrosine residue essential for activation, Y699, is located in the region between the SH2 and TAD domains.


STAT5b
Y699
1
144
330
496
592
685
707
787
N-term
CC
DBD
SH2
TAD

The coiled-coil (CC) domain is comprised of 4 antiparallel alpha helices which participate in protein-protein interactions (Horvath et al., 1996). These interactions are involved in receptor recruitment, demonstrated by co-immunoprecipitation of the IL-6 receptor with the coiled-coil domain of STAT3 (Zhang et al., 2000).

The DNA binding domain (DBD) is an immunoglobulin-type fold that forms a "C"-shaped clamp around DNA, structurally similar to the DNA binding domains of p53 and NFκB transcription factors (Chen et al., 1998; Cho et al., 1994; Ghosh et al., 1995). Crystallization studies of the STAT1 DBD showed that the DBD contacts a 15 base pair region of the DNA, but does not make direct contact with the phosphate backbone (Chen et al., 1998).

The linker region is believed to interact with transcriptional machinery, based on the observation that point mutations in the STAT1 linker region prevent cytokine-induced transcription but do not affect tyrosine phosphorylation, dimerization, nuclear translocation, or DNA binding (Yang et al., 1999).

The SH2 domain is essential for formation of active STAT dimers. This occurs through a reciprocal interaction between the conserved phosphorylated tyrosine of one monomer and the SH2 domain of another (Chen et al., 1998; Heim et al., 1995). The SH2 domain also allows for association of STATs with phosphorylated tyrosines on receptors. A single point mutation in the SH2 domain of STAT2 prevents its binding to the IFNα receptor (Li et al., 1997). Additionally, exchanging SH2 domains between STAT proteins alters receptor binding (Heim et al., 1995).

The transactivation domain (TAD) is less than 100 amino acids at the extreme C-terminus of the protein. It is the least conserved domain and confers specificity in gene regulation. The structure of this domain is very flexible, allowing interaction with multiple binding partners such as transcriptional coactivators (Paulson et al., 1999; Pfitzner et al., 1998).

The TAD contains a number of tyrosine and serine residues whose phosphorylation modulates transcriptional activity (Decker and Kovarik, 2000; Kloth et al., 2002; Olayioye et al., 1999; Weaver and Silva, 2006; Weaver and Silva, 2007a).

Lastly, each STAT protein contains a conserved tyrosine residue essential for activation (Darnell, 1997; Gouilleux et al., 1994; Lin et al., 1996). This tyrosine is located between the SH2 and TAD domains.

STAT Signaling

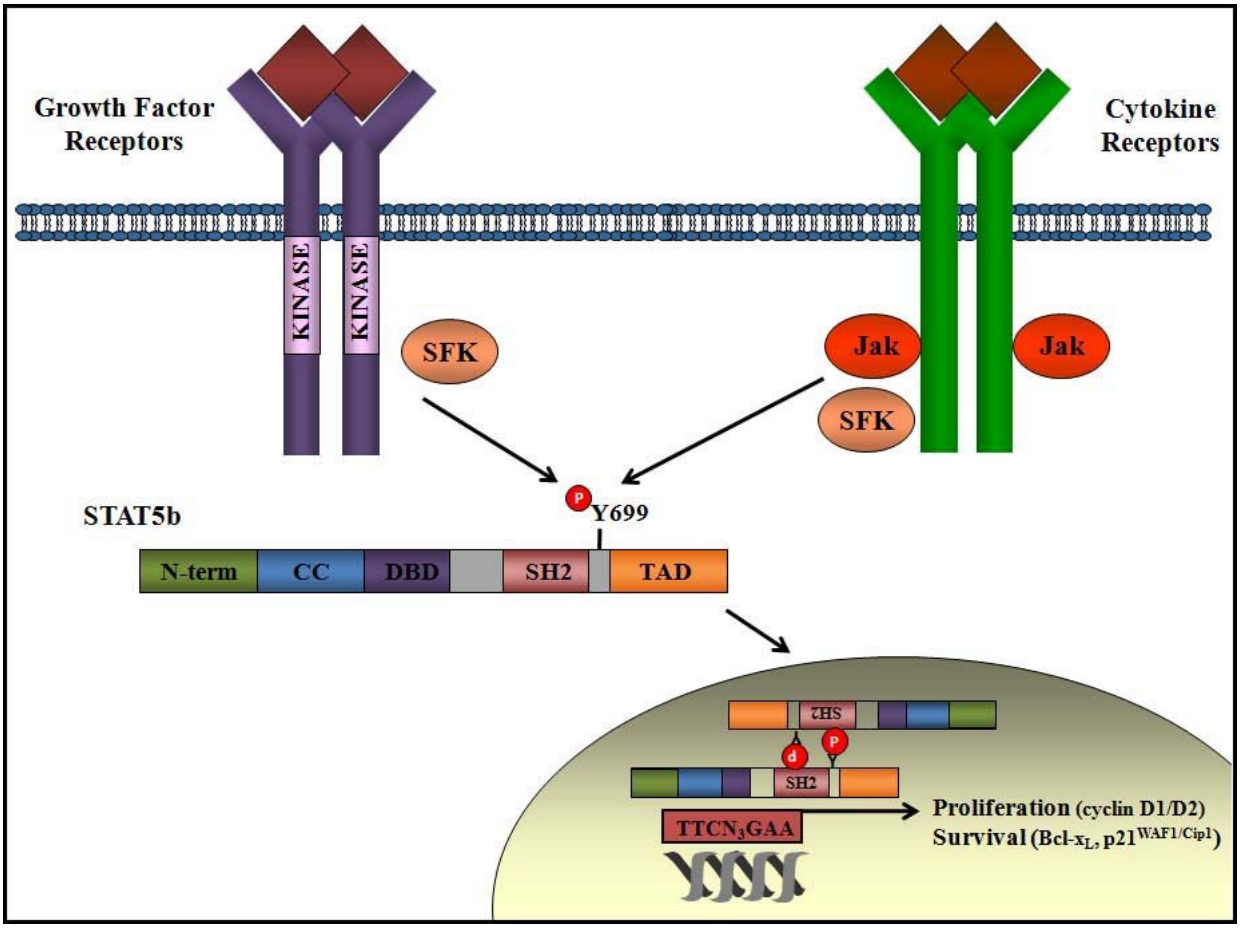
Canonical Signaling

All STAT proteins are activated in a similar manner. Figure 2 depicts the activation of STAT5b downstream of either growth factor or cytokine receptors. Upon ligand binding and subsequent receptor activation, STAT proteins are recruited to the receptor and phosphorylated on the conserved tyrosine (Y699 for STAT5b). Once phosphorylated, active dimers form via a reciprocal interaction between this phosphorylated tyrosine and the SH2 domain arginine (R618 for STAT5b) of the other STAT. Active dimers translocate to the nucleus and bind promoters of target genes.

It is not completely clear how STAT dimers enter the nucleus, as they do not contain a classic nuclear localization signal (NLS), defined by a cluster of basic amino acids. However, STAT dimers are found in the nucleus following stimulation, leading some to argue that STAT proteins contain a cryptic NLS which is exposed upon phosphorylation (Milocco et al., 1999; Pranada et al., 2004; Sekimoto et al., 1996; Xu and Massague, 2004; Zeng et al., 2002). Others have reported that NLS-containing receptors, including IFNγR and HER4, can function as

Figure 2: STAT5b Signaling

This figure illustrates the canonical signaling pathway for STAT5b downstream of both growth factor receptors and cytokine receptors. As depicted on the left, once ligand binds to receptor, the receptor is phosphorylated on tyrosine residues which serve as docking sites for STAT5b. The receptor, and/or nonreceptor tyrosine kinases, such as Src family kinases (SFKs), can then phosphorylate Y699 of STAT5b. On the right, Janus kinases (JAK) associate with ligand-bound cytokine receptors leading to activation of JAKs. Activated JAKs can then phosphorylate Y699 of STAT5b. Once STAT5b is phosphorylated, it forms active dimers through pY-SH2 interactions and translocates to the nucleus where it binds consensus promoter sites, leading to transcription of target genes involved in proliferation and survival.


Growth Factor Receptors
Cytokine Receptors
KINASE
KINASE
SFK
Jak
Jak
SFK
P
Y699
STAT5b
N-term
CC
DBD
SH2
TAD
SH2
P
d
SH2
TTCN3GAA
Proliferation (cyclin D1/D2)
Survival (Bcl-xL, p21WAF1/Cip1)

chaperones and shuttle STAT proteins into the nucleus (Subramaniam et al., 2001; Williams et al., 2004). Alternatively, there is evidence that unphosphorylated STATs directly associate with nuclear import proteins. The STAT1 linker domain associates with nucleoporins 153 and 214 independent of cytokine or growth factor stimulation (Marg et al., 2004). Also, the STAT3 CC domain is critical for association with importin-α3, and this association occurs independent of STAT3 phosphorylation (Ushijima et al., 2005). Regardless of how STAT proteins enter the nucleus, once there, STAT dimers bind TTC(N3)GAA γ-interferon-activating sequence (GAS) sites in the promoters of target genes, regulating gene transcription (Ihle, 1996; Liu et al., 1995; Udy et al., 1997). STATs regulate numerous genes involved in proliferation, cell cycle progression, and survival including cyclin D1, c-myc, $p21^{WAF1/CIP1}$, and Bcl-x_L (Bowman et al., 2000).

Unphosphorylated Dimer Signaling

For many years, it was believed that unphosphorylated STAT proteins exist in the cytoplasm strictly as monomers, and only form dimers once phosphorylated. However, in recent years, unphosphorylated STAT dimers have been found in both the cytoplasm and nucleus (Kretzschmar et al., 2004; Mao et al., 2005; Ndubuisi et al., 1999; Neculai et al., 2005; Schroder et al., 2004; Zhong et al., 2005). Cytoplasmic STAT3 homodimers were isolated from myeloid progenitor cells (Novak et al., 1998), and STAT1-STAT3 heterodimers were co-immunoprecipitated from unstimulated hepatoma, melanoma, and cervical cancer cell lines (Haan et al., 2000; Stancato et al., 1996). Unphosphorylated STATs in the cytoplasm are not simply latent proteins, but rather can be actively involved in cellular processes. For example,

unphosphorylated STAT3 regulates microtubule dynamics by binding to the microtubule destabilizing protein stathmin, preventing it from depolymerizing tubulin (Ng et al., 2006).

Unphosphorylated STATs are also found in the nucleus. Nuclear staining of tyrosine mutated (YF) STAT1 and STAT3 has been reported in numerous cell types (Meyer et al., 2002). Interestingly, overexpression of Y701F STAT1 or Y705F STAT3 induces gene transcription (Yang et al., 2005). While the exact mechanism of this transcriptional activity is unknown, it is believed to be independent of DNA binding and similar to transcriptional coactivators.

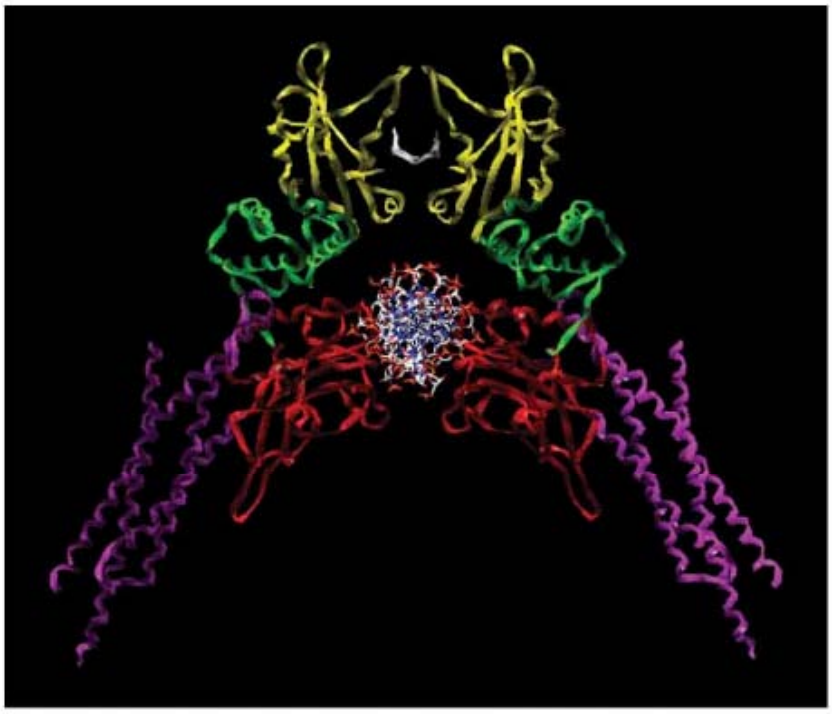
X-ray crystal structure analysis has been performed for both phosphorylated (STAT1 and STAT3) and unphosphorylated (STAT1 and STAT5a) dimers from a "core fragment" containing the CC, DBD, linker, and SH2 domains (Becker et al., 1998; Chen et al., 1998; Mao et al., 2005; Neculai et al., 2005). Due to the flexibility of the N-terminus and C-terminal TAD, these regions have not been crystallized. Figure 3 depicts the current crystal structure models. Phosphorylated dimers take on a parallel conformation due to reciprocal pY-SH2 interactions described earlier. The short region connecting the SH2 and TAD domain is thought to facilitate this conformation because it is flexible enough to allow association between the phosphorylated tyrosine of one monomer and the SH2 domain of the other, but is too short for the phosphorylated tyrosine to bind its own SH2 domain (Cao et al., 2006). When bound to DNA, the phosphorylated dimer forms a "nutcracker" structure with the DBDs flanking the DNA (Figure 3B). In contrast, unphosphorylated dimers take on an antiparallel "boat-like" configuration (Figure 3C). This association is weaker than the phosphorylated dimer, but is stabilized by interactions between the DBDs (Mao et al., 2005; Neculai et al., 2005). In this conformation, the SH2 domains are on opposite sides of the structure and are free to interact with phosphorylated tyrosines of other proteins. The difference in these structures demonstrates that STAT dimers undergo a major

Figure 3: Structure of Phosphorylated and Unphosphorylated STAT Dimers

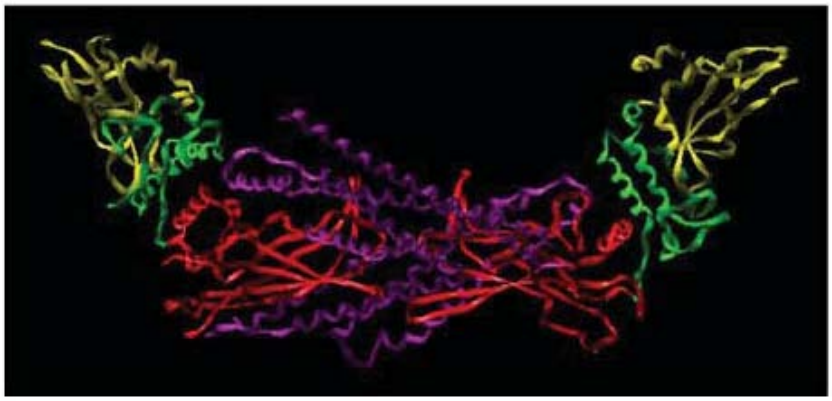
(A) Domain structure of STAT proteins, color-coded to match dimer structures: CC (*purple*), DBD (*red*), linker (*green*), SH2 (*yellow*). (B) Crystal structure of phosphorylated STAT dimer bound to DNA. DNA is depicted as white/blue helix located between the DBDs. Conserved phosphotyrosine tail is depicted as short white segment proximal to the SH2 domain. (C) Crystal structure of unphosphorylated antiparallel STAT dimer.

A

B



C



structural rearrangement upon phosphorylation. This change may be facilitated by the flexibility of the N- and C- termini.

STAT5a and STAT5b

Research in our lab has primarily focused on two STAT family members, STAT5a and STAT5b. STAT5a and STAT5b are encoded by separate genes which will be described in more detail later. These genes are 95% homologous and are believed to have arisen from gene duplication (Wittig and Groner, 2005). Despite their sequence similarities, STAT5a and STAT5b exhibit distinct functions during development, best illustrated by knockout studies. Both STAT5a and STAT5b knockout mice are fully viable. $STAT5a^{-/-}$ mice are similar in size and weight to wild-type littermates, and are fertile (Liu et al., 1997). However, $STAT5a^{-/-}$ mice exhibit defective lobuloalveolar outgrowth during pregnancy and cannot lactate (Liu et al., 1997). STAT5b knockout mice undergo normal lactogenesis, but display altered growth patterns. $STAT5b^{-/-}$ male mice are similar in size to wild-type female mice and $STAT5b^{-/-}$ female mice are larger than their wild-type littermates (Udy et al., 1997). In summary, STAT5a is a mediator of Prl signaling and is involved in development of the mammary gland, whereas STAT5b mediates GH signaling and is an important regulator of sexual dimorphism (Liu et al., 1997; Udy et al., 1997). The sequence diversity that exists between STAT5a and STAT5b is primarily located in the TAD and thus, most likely contributes to their unique roles in cellular signaling.

Since STAT5a and STAT5b bind to the same GAS consensus sites, one might expect that they bind to the same target gene promoters. Both bind an assortment of gene promoters including β-casein, IL-2Rα, $p21^{WAF1/Cip1}$, oncostatin M (OSM), serine protease inhibitor 2.1

(Spi2.1), and cytokine-inducible SH2-domain-containing protein (CIS) (Ehret et al., 2001; Verdier et al., 1998). However, STAT5a and STAT5b exhibit different binding specificities and affinities depending on the target gene and cellular environment (Soldaini et al., 2000; Verdier et al., 1998).

STATs and Cancer

In view of the fact that STAT proteins regulate genes controlling cell proliferation, differentiation, and survival, it is no surprise that STAT activation is linked to tumorigenesis. The major findings implicating STAT signaling in cancer include: (1) tyrosine kinases frequently overexpressed or activated in tumors phosphorylate/activate STATs; (2) constitutive activation of STATs induces cellular transformation; and (3) inhibition of STAT-mediated transcription inhibits kinase-induced cellular transformation (Bowman et al., 2000). In particular, activation of STATs 1, 3, 5a, and 5b occurs in a variety of cancers, both hematopoietic cancers and solid tumors such as those of the breast, prostate, lung, head and neck, and brain (Table 1) (Bowman et al., 2000; Turkson and Jove, 2000). While STAT1 appears to function primarily as a tumor suppressor, activation of STATs 3, 5a, and 5b promotes tumorigenesis (Calo et al., 2003; Haura et al., 2005; Huang et al., 2002; Tan and Nevalainen, 2008; Widschwendter et al., 2002).

STAT1

The first evidence that STAT1 functions as a tumor suppressor came from a study reporting that mice lacking STAT1 have a higher incidence of tumor formation following exposure to carcinogens, and a higher rate of spontaneous tumor formation in the absence of p53 (Kaplan et al., 1998). *In vitro*, STAT1 signaling induces cell cycle arrest and apoptosis

Table 1: Activated STAT Proteins in Cancer

Activated STAT Proteins in Cancer

Tumor Type	Activated STAT
Solid Tumors	
Breast	STAT1, STAT3, STAT5
SCCHN	STAT1, STAT3, STAT5
Melanoma	STAT3, STAT5
Prostate	STAT3, STAT5
Lung	STAT3, STAT5
Ovarian	STAT3, STAT5
Pancreatic	STAT3, STAT5
Colon	STAT3, STAT5
Renal	STAT3, STAT5
Glioblastoma	STAT1, STAT3
Hematopoietic - Leukemia	
Acute lymphocytic (ALL)	STAT1, STAT5
Chronic lymphocytic (CLL)	STAT1, STAT3
Acute myelogenous (AML)	STAT1, STAT3, STAT5
Chronic myelogenous (CML)	STAT5
Large granular lymphocytic (LGL)	STAT3
Erythroleukemia	STAT1, STAT5
Hematopoietic - Lymphoma	
EBV-related/Burkitt's	STAT3
Cutaneous T-cell	STAT3, STAT5
Hodgkin's	STAT3, STAT5
Non-Hodgkin's	STAT3

Adapted from Bowman, et al., 2000; Yu, et al., 2004

(Haura et al., 2005). However, results from analysis of STAT1 primary tumor samples are not as clear-cut as *in vitro* and *in vivo* data. While decreased levels of STAT1 are common in squamous cell carcinoma of the head and neck (SCCHN) tumors, increased levels of STAT1, as well as tyrosine and serine phosphorylation of STAT1, are found in leukemia and cervical cancer specimens (Frank et al., 1997; Hudelist et al., 2005; Spiekermann et al., 2001; Xi et al., 2006). Therefore, STAT1's role in tumorigenesis may be cell-type and context dependent.

STAT3

STAT3 is overexpressed and/or constitutively activated in breast, ovarian, SCCHN, and prostate cancers (Berclaz et al., 2001; Giri et al., 2001; Li and Shaw, 2002; Silver et al., 2004). In breast cancer cells, STAT3 signaling promotes proliferation and survival, and constitutive activation of STAT3 correlates with EGFR and c-Src activity (Bromberg, 2002). STAT3 levels are upregulated in invasive ovarian cancer and loss of STAT3 inhibits *in vitro* migration (Silver et al., 2004). In SCCHN, constitutive STAT3 increases survival and introduction of dominant-negative STAT3 inhibits growth (Grandis et al., 1998; Grandis et al., 2000). Similar to breast, ovarian, and SCCHN cells, STAT3 stimulates proliferation and survival in prostate cancer cell lines (Barton et al., 2004; Lee et al., 2003; Mora et al., 2002). Constitutive STAT3 phosphorylation is associated with advanced stage prostate cancer and tyrosine phosphorylated STAT3 is present in lymph node and bone metastases (Abdulghani et al., 2008; Horinaga et al., 2005; Mora et al., 2002). Accordingly, STAT3 promotes prostate cancer motility and increases frequency of lung metastases *in vivo* (Abdulghani et al., 2008).

STAT5a and STAT5b

Increased STAT5a/b phosphorylation is seen in a variety of solid tumors and epithelial carcinomas including SCCHN, lung, prostate, and breast cancers (Bowman et al., 2000; Calo et al., 2003; Haura et al., 2005; Yu and Jove, 2004). Due to their highly conserved sequences, phospho-specific STAT5 antibodies recognize both STAT5a and STAT5b. However, STAT5b is the predominant STAT5 protein expressed in cancer cell lines and *in vitro* studies support a pro-tumorigenic function for STAT5b specifically (Kazansky et al., 2003; Weaver and Silva, 2006; Xi et al., 2003b). While both STAT5a and STAT5b are activated by v-Src, only STAT5b accelerates v-Src-mediated transformation of fibroblasts (Kazansky and Rosen, 2001). In these cells, STAT5b increases v-Src-induced growth, survival, and motility. Antisense inhibition of STAT5b, but not STAT5a, inhibits growth of SCCHN xenografts (Xi et al., 2003a). Additionally, dominant-negative STAT5b inhibits proliferation, invasion, and *in vivo* tumor growth of metastatic prostate cancer cells (Kazansky et al., 2003). In breast cancer, STAT5b, but not STAT5a, mediates cellular proliferation and survival (Fox et al., 2008; Kloth et al., 2003; Weaver and Silva, 2006).

Cell Migration

Hanahan and Weinberg outlined six properties of cancer cells that allow for tumorigenesis, one of which is tissue invasion and metastasis (Hanahan and Weinberg, 2000). In order to metastasize, cells must dissociate from their primary site, enter into circulation, exit into the secondary site, and ultimately colonize the new tissue (Chambers et al., 2002). Cell migration is essential for multiple steps in this cascade. Migration is a complex process

involving cell-matrix interactions, cytoskeletal changes, actin-myosin contractions, and assembly/disassembly of focal contacts (Friedl and Wolf, 2003; Gupta and Massague, 2006).

When a cell senses a migratory stimulus, it undergoes cytoskeletal rearrangements which allow the cell to polarize and extend lamellipodial protrusions toward the stimulus. These protrusions are stabilized by attachment to extracellular matrix (ECM) components (Ridley et al., 2003). Once a cell has protruded and attached, the cell body contracts pulling the cell forward, and rear adhesions are disassembled, resulting in directional movement (Ridley et al., 2003). There are numerous signaling proteins involved in each of these processes. The Rho family of small GTPases and adhesion-dependent kinases play a major role in this signaling and are discussed in detail below.

Polarization

The Rho family of small GTP-binding proteins (RhoA, Rac, Cdc42) is critical for many aspects of cell migration, including cell polarity. Activation of small G proteins is a cyclic process (Ridley, 1995). In their inactive state, small G proteins are bound to GDP. In order to become active, these proteins are recruited to the membrane where guanine nucleotide exchange factors (GEFs) replace GDP with GTP. GTP-bound proteins can then engage downstream effector proteins and regulate various biological pathways (Etienne-Manneville and Hall, 2002). When the bound GTP is hydrolyzed to GDP by GTPase-activating proteins (GAPs), the small G protein returns to its inactive state.

In migrating cells, the Rho GTPase Cdc42 localizes to the front of migrating cells and is responsible for re-orientation of the microtubule-organizing center (MTOC) (Itoh et al., 2002). The MTOC re-orients between the nucleus and leading edge during migration, allowing MT-

mediated protein delivery to the leading edge (Etienne-Manneville and Hall, 2002). The Rho GTPase Rac also localizes to the front of the cell where it stabilizes MTs, regulates protrusion formation, and maintains cell polarity (Rodriguez et al., 2003). The mechanisms responsible for maintaining cell polarity during migration are not completely understood, but one model suggests that it results from Rho-Rac antagonism (Evers et al., 2000; Ridley et al., 2003). Rac expression and activity is higher in the front of cells where it regulates protrusion signaling at the leading edge. Rho activity, on the other hand, predominates at the sides and rear of the cell where it regulates contraction and tail retraction (Ridley et al., 2003).

Membrane Protrusion: Actin Dynamics

Actin Polymerization

Membrane protrusion at the leading edge is a result of actin filament assembly at the membrane. Actin monomers can form dimers or trimers. Dimer association is weak, but addition of a third monomer stabilizes the complex, allowing for formation of a nucleation site and subsequent elongation. Elongated actin filaments are comprised of a fast-growing barbed end, and a slow-growing pointed end. The barbed end is oriented toward the membrane and filament elongation at this end pushes the membrane outward. The rate of elongation is dependent on the availability of free actin monomers (Pollard and Borisy, 2003).

Additionally, filament assembly is regulated by several actin-binding proteins. The Arp2/3 complex of proteins promotes nucleation of pre-existing actin filaments, creating branched filaments (Pollard and Borisy, 2003). Arp2/3 is intrinsically inactive and requires activation by the Cdc42-activated protein Wiskott-Aldrich syndrome family protein (WASP) (Rohtagi et al., 1999). In addition to the Arp2/3-WASP complex, profilin also stimulates

filament assembly. Profilin associates with actin filaments, stimulating elongation of barbed ends while blocking monomer association with pointed ends (Pollard and Borisy, 2003). Capping proteins also regulate actin assembly by binding to ("capping") the barbed end, preventing further elongation (Isenberg et al., 1980). Depolymerization is regulated by ADF/cofilin which can either sever actin filaments (Ichetovkin et al., 2000) or increase the rate of monomer dissociation at the pointed end (Carlier et al., 1997).

Contraction

Actomyosin contraction is essential for cell motility. Contraction of actin filaments produces tension across the cell, pulling on the ECM to which the cell is attached, and facilitating movement along the substratum. This contraction is mediated by myosin. Myosins are motor proteins which bind to, and move along, actin filaments using ATP hydrolysis. Non-muscle myosin II is the primary motor protein responsible for promoting motility of eukaryotic cells (Bresnick, 1999). Mammalian cells contain three isoforms of myosin II (IIA, IIB, IIC). These isoforms differ in the composition of their heavy chains and display unique tissue expression patterns (Eddinger and Meer, 2007; Golomb et al., 2004). All myosin II family members are composed of two heavy chains and four light chains (two regulatory and two essential) which together form a structure containing a "head", "neck", and "tail" (Eddinger and Meer, 2007). The myosin heads bind actin filaments and ATP, and are the motor domains. The neck serves as a lever arm transducing force generated by the catalytic head domain. Myosin tails bind cargo necessary for facilitating this activity.

Actomyosin contraction occurs through myosin-mediated sliding of two antiparallel actin filaments. This process is regulated by phosphorylation of the two myosin regulatory light

chains (MLCs) located at the neck of the structure (Ikebe, 2008). Inactive myosin takes on a closed, bent structure in which the motor head associates with the tail. MLC can be phosphorylated by multiple kinases (*discussed below*). Phosphorylation of MLC results in a structural change to an open, extended conformation allowing actin binding and motor activity. MLC is dephosphorylated by myosin light chain phosphatase (MLCP), reverting MLC to its inactive conformation.

The two major phosphorylation sites on MLC are Threonine 18 (T18) and Serine 19 (S19). Myosin light chain kinase (MLCK) is the primary kinase that phosphorylates these two sites (Gallagher et al., 1997). However, there are several additional mechanisms by which phosphorylation of MLC can occur. Activated Rac stimulates p21-associated kinase (PAK) activity which directly phosphorylates S19 of MLC (Chew et al., 1998). PAK also phosphorylates and inactivates MLCK, resulting in decreased MLC phosphorylation (Sanders et al., 1999). Activated RhoA stimulates Rho-kinase (ROCK) which phosphorylates MLCP (Kimura et al., 1996). This phosphorylation inactivates MLCP, resulting in increased MLC phosphorylation. Recent work suggests that ROCK may dually phosphorylate S19/T18 of MLC (Hirata et al., 2009). However, direct phosphorylation of myosin II by ROCK was not shown, and the possibility of MLC phosphorylation by a yet unidentified kinase downstream of ROCK remains (Hirata et al., 2009).

Current models maintain that monophosphorylation of S19 increases myosin activity, and that subsequent T18 phosphorylation maximizes this activity (Ikebe, 2008). However, a recent study identified differential functions of mono- and di- phosphorylated myosin II in spreading of human lung fibroblasts (Hirata et al., 2009). During initial stages of spreading, cells form a ring-like structure of F-actin from which lamellipodia extend. The study by *Hirata, et al.,* found that

both mono- and di- phosphorylated myosin II localized within the actin ring, but only diphosphorylated myosin II was localized in the lamellar region (Hirata et al., 2009). Treatment of cells with ROCK inhibitor led to loss of diphosphorylated myosin II, decreased actin ring formation, increased spreading, and abnormal cell shape. These data indicate that diphosphorylation of myosin II is essential for proper cell spreading (Hirata et al., 2009).

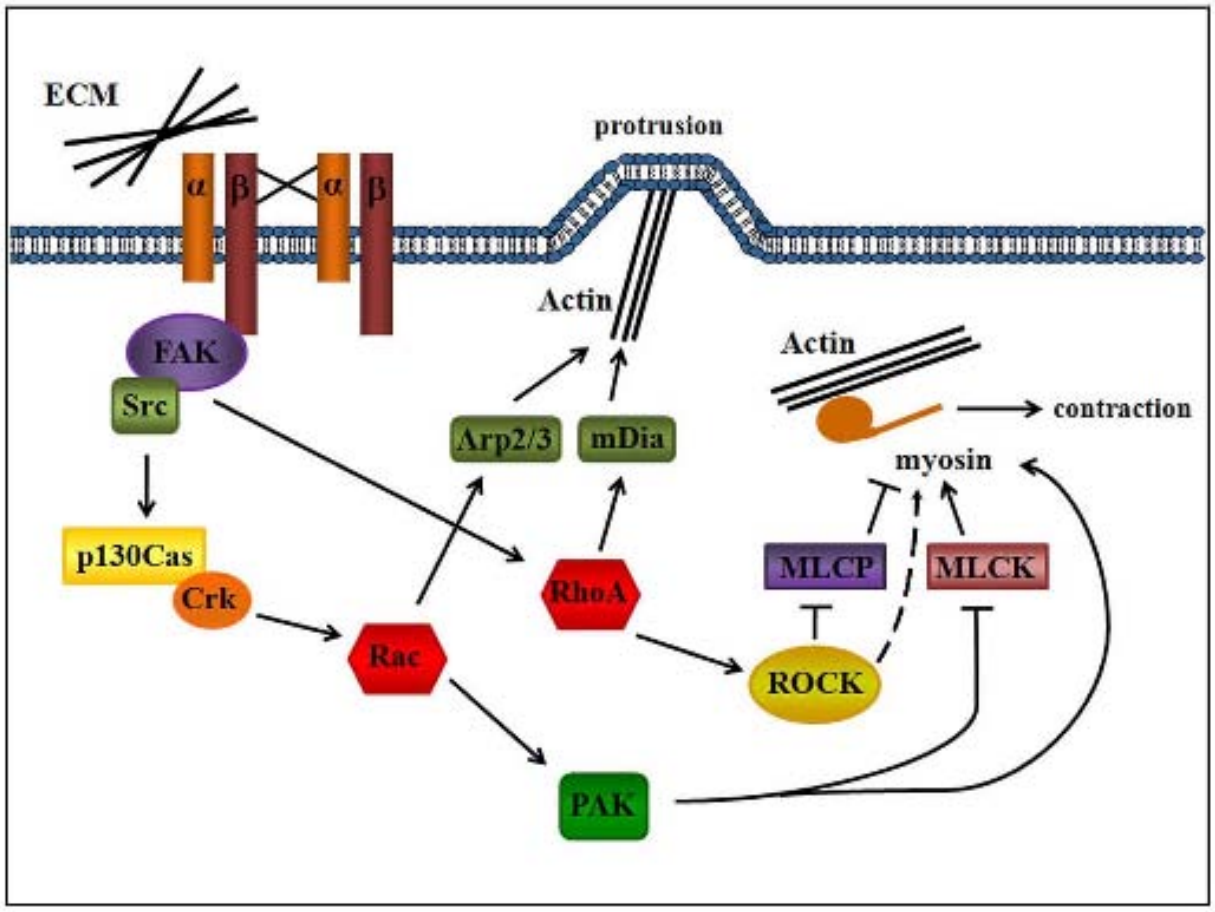
Membrane Protrusion - Adhesion Dynamics

Lamellipodia extension requires turnover of cellular adhesions. Cellular adhesions link the cytoskeleton to the ECM, mediating signal transmission from the cellular environment into the cell (Friedl and Wolf, 2003). During migration, adhesions are continuously being assembled and disassembled. Initially, small focal adhesion complexes form at the leading edge of cells. Over time these complexes become larger and translocate under the cell body, stabilizing cell attachment to the ECM. These larger complexes are referred to as focal adhesions. At the same time, new focal complexes are forming at the leading edge and the process continues (Webb et al., 2003).

Adhesion assembly and disassembly involves integrin and focal adhesion kinase (FAK) signaling. Integrins are cell surface receptors that bind to ECM components, transducing extracellular signals into the cell. They are transmembrane heterodimers composed of one alpha (α) and one beta (β) subunit, and can bind numerous ligands including fibronectin, vitronectin, collagen, and laminin (Hynes, 2002). Once bound to ligand, integrins associate and recruit additional proteins initiating signaling cascades within the cell (reviewed in Huveneers and Danen, 2009) (Figure 4).

Figure 4: Migratory Signaling Pathways

Following integrin engagement with the ECM, integrin receptors cluster and recruit proteins to the membrane, including FAK and Src kinases. FAK and Src signaling result in activation of the small GTPases Rac and RhoA. Rac activates PAK promoting adhesion turnover and regulating contractility. PAK phosphorylation of myosin stimulates actomyosin contraction but PAK can also phosphorylate and inactivate MLCK, suppressing contraction. RhoA activates ROCK which stimulates contractility by inhibiting MLCP or potentially through direct phosphorylation of MLC (*dashed line*). RhoA and Rac also regulate protrusion through activation of the nucleating proteins Arp2/3 and mDia.


ECM
protrusion
α
β
α
β
Actin
FAK
Src
Arp2/3
mDia
Actin
contraction
myosin
p130Cas
Crk
RhoA
MLCP
MLCK
Rac
ROCK
PAK

Two important proteins recruited to integrin complexes are the non-receptor tyrosine kinases FAK and c-Src. FAK association with integrins results in FAK autophosphorylation on Y397. c-Src binds phosphorylated Y397 and phosphorylates additional tyrosine residues on FAK, increasing FAK kinase activity and providing binding sites for additional proteins, many of which are FAK-Src substrates (Cobb et al., 1994). One such protein is the adaptor protein p130Cas/BCAR1. Phosphorylated p130Cas associates with the adaptor protein Crk, leading to recruitment of GEFs which activate Rac. Phosphorylation of paxillin by the FAK-Src complex also leads to recruitment of GEFs for Rac as well as Cdc42. Additionally, Src can phosphorylate p190RhoGAP, inhibiting RhoA activity. These combined interactions promote membrane protrusion and adhesion turnover (Huveneers and Danen, 2009).

Tail Retraction

Tail retraction requires disassembly of focal contacts at the rear of the cell, which can occur through several mechanisms (Vicente-Manzanares et al., 2005). The first mechanism is depolymerization of actin filaments by the actin-binding protein ADF/cofilin mentioned earlier (Carlier et al., 1997; Ichetovkin et al., 2000). Additionally, phosphatases such as protein tyrosine phosphatase α (PTPα) can dephosphorylate adhesion complexes, disrupting adhesion signaling (Zeng et al., 2003). Lastly, proteases such as calpain cleave components of the adhesion complexes, resulting in detachment from the ECM (Pfaff et al., 1999).

STATs and Cell Migration

While STATs are primarily thought of as transcription factors that regulate cell growth and survival, a role for STATs in cell motility is taking shape. STAT3 and STAT5b have been

implicated in prostate tumor cell migration and invasion (Abdulghani et al., 2008; Kazansky et al., 2003; Zhou et al., 2006). Both STAT proteins have been found in migratory structures. Phosphorylated STAT3 is present in pseudopods and focal adhesions (Jia et al., 2005; Silver et al., 2004). In focal adhesions, STAT3 is associated with phosphorylated FAK and paxillin (Silver et al., 2004). Phosphorylated STAT5b localizes to podosomes in leukemia cells (Poincloux et al., 2007). It is of note that STAT5b does not localize to podosomes in human primary macrophages, suggesting this may be specific to transformed cells (Poincloux et al., 2007). It is not known how STAT3 and STAT5b are signaling at these sites, but there is a body of work implicating these proteins in regulation of the cytoskeleton. STAT3 expression polarizes actin and microtubules (MTs) and induces lamellipodia formation in prostate cancer cells (Abdulghani et al., 2008). A recent study in fibroblasts found that unphosphorylated STAT3 indirectly regulates MT dynamics by associating with and inhibiting the function of the MT depolymerizing protein stathmin (Ng et al., 2006). STAT3 binds the C-terminus of stathmin, the same region bound by tubulin. Thus, STAT3 may be inhibiting MT depolymerization by competing with tubulin for stathmin binding (Gao and Bromberg, 2006; Ng et al., 2006).

In breast cancer cells, STAT5b, but not STAT3 or STAT5a, associates with MTs (Phung-Koskas et al., 2005). STAT5b preferentially associates with dynamic MTs and not their stable counterparts. Stabilization of MTs leads to loss of GH-induced STAT5b nuclear translocation, suggesting that STAT5b may utilize MTs for transport into the nucleus (Phung-Koskas et al., 2005). However, STAT5b association with MTs does not require Y699 phosphorylation. Therefore, unphosphorylated STAT5b bound to MTs may regulate MT dynamics and subsequent motility (Liao et al., 1995).

Additionally, STAT3 and STAT5a can be activated downstream of Rho GTPase signaling, supporting a potential role for STATs in migration. In kidney cells, constitutively active RhoA induces JAK2-mediated phosphorylation and transcriptional activity of both STAT3 and STAT5a, but has no effect on STAT5b phosphorylation (Aznar et al., 2001; Benitah et al., 2003). RhoA-mediated activation of STAT3 also occurs in Rat1 fibroblasts, and STAT3 null fibroblasts show decreased RhoA-mediated proliferation, stress fiber formation, and migration (Debidda et al., 2005; Faruqi et al., 2001). In mammary epithelial cells, dominant-negative forms of either Rac1 or cdc42 suppress Prl-induced STAT5a phosphorylation and nuclear translocation, suppressing differentiation (Akhtar and Streuli, 2006).

Research Goals

Breast cancer is the second leading cause of cancer deaths among women, with the majority of deaths resulting from metastatic disease. There are no effective treatments to prevent metastasis and the five year survival rate for women diagnosed with distant disease is less than 25% [American Cancer Society]. Current targeted therapies have not been as successful as anticipated. This lack of success is due in part to the ability of cancer cells to upregulate alternative signaling pathways to promote tumor growth and progression. Many tumorigenic signaling pathways converge on common nuclear transcription factors and therefore, targeting these downstream proteins may be more effective. One such group of transcription factors is the STAT family of proteins. STAT5b, a member of the STAT family, is overexpressed and activated in numerous cancers, including breast cancer. STAT5b promotes proliferation and survival of breast cancer cells downstream of both ER and EGFR/c-Src signaling (Boerner et al., 2004; Fox et al., 2008; Kloth et al., 2002; Kloth et al., 2003; Weaver and Silva, 2006).

Approximately 40-70% of all breast tumors are ER-positive (ER+) (McGuire, 1975). In general, ER+ tumors are less aggressive than their ER-negative counterparts, and patients with ER+ tumors benefit from the availability of antiestrogen targeted therapies, including tamoxifen. Unfortunately, some patients do not respond to these therapies and others acquire resistance over time (Clarke et al., 2003). One proposed mechanism accounting for tamoxifen resistance is upregulation of EGFR family members, c-Src, and the adaptor protein Cas, which provide alternative proliferative pathways (Riggins et al., 2006; Silva and Shupnik, 2007). STAT5b is a downstream effector of these signaling pathways and can mediate tamoxifen resistance. In ER+ breast cancer cells, dominant-negative STAT5b inhibits Cas-mediated tamoxifen resistance, and expression of constitutively active STAT5b alone confers tamoxifen resistance (Fox et al., 2008; Riggins et al., 2006). ER negative tumors often overexpress members of the EGFR family along with c-Src (Belsches-Jablonski et al., 2001; Biscardi et al., 1998). EGFR overexpression is characteristic of highly aggressive tumors and poor clinical outcome (Nielsen et al., 2004). In tumors that co-overexpress EGFR and c-Src, c-Src phosphorylates tyrosine 845 on the EGFR, leading to downstream activation of STAT5b and STAT5b-mediated DNA synthesis and survival (Kloth et al., 2003).

While it is clear that STAT5b mediates proliferation and survival of both ER+ and EGFR-overexpressing breast tumors, there is limited work on its contribution to other tumorigenic processes such as migration and invasion. These processes are essential for tumor cells to metastasize. Thus, the objective of this thesis was to determine the importance of STAT5b for breast cancer cell migration. Ascertaining the role of STAT5b in breast cancer cell migration may facilitate the development of more effective therapies for the prevention and treatment of metastatic disease.

Body

Task 1: Investigate the role of STAT5b in breast cancer cell migration

A. Determine whether loss of STAT5b affects breast cancer cell migration

Establishment and Optimization of a Breast Cancer Model System

Initially, we examined the migratory ability of a panel of EGFR-expressing breast cancer cell lines using a transwell system. In this system, cells are plated onto a filter in an upper chamber suspended over a second, lower chamber. The filter contains pores large enough for cells to pass through. Chemoattractants, such as serum, growth factors, or chemokines, are placed in the lower chamber and the ability of cells to transmigrate through the filter is assessed. Figure 5A shows the average migration of BT-20, MDA-MB-468, SKBr3, BT-549, MDA-MB-231, 21PT, and 21MT-1 human breast cancer cell lines to serum over six hours. The amount of migration varied between cell lines, but was highest in BT-549 cells. Therefore, we chose the BT-549 cells as our primary model system for further studies. BT-20, MDA-MB-231, and 21PT cells also migrated well to serum. Of these three cell lines, we chose the MDA-MB-231 cell lines as a secondary system because similar to BT-549 cells, they overexpress the EGFR, are stromal-like, and have high invasive potential (Lacroix and Leclercq, 2004). BT-20 cells also express high levels of EGFR, but are luminal-like and have weak invasive potential. 21PT cells were not chosen because this cell line is not well established in the literature.

As seen in Figure 5B, BT-549 cells migrated extremely well to 10% serum. To allow for accurate counting, we needed to optimize our system. First, we varied the amount of cells plated in the upper chamber of the transwell plate (Figure 6A). Very little migration was observed using $1x10^4$ cells. However, approximately the same amount of migration occurred when plating $5x10^4$ or $1x10^5$ cells, indicating that maximal migration could be achieved with half the amount

Figure 5: Establishment of a Breast Cancer Cell Model System

(A) Table depicting average migration counts for BT-20, MDA-MB-468, SKBr3, BT-549, MDA-MB-231, 21PT, and 21MT-1 human breast cancer cell lines to serum. For each cell line, $1x10^5$ cells were plated in serum-free media (DMEM + 0.1% BSA) in upper chambers of transwell migration plates. Serum-containing media (DMEM + 10% FBS) was placed in lower chambers to serve as a chemoattractant. After 6 hours, non-migratory cells were removed with a cotton swab and remaining cells were fixed in methanol and stained with 0.1% crystal violet in 20% ethanol. Four fields were counted on each of two filters. Results are listed as average number of migratory cells per field for one experiment. (B) Comparison of the two cell lines chosen for future studies, BT-549 and MDA-MB-231 (*gray shading in part A*). Graph illustrates migration to serum-free media (SF) vs. serum-containing media (10% FBS) over 6 hours.

A

Breast Cancer Cell Line	Average Migratory Cells/Field
BT-20	70
MDA-MB-468	21
SKBr3	4
BT-549	**228**
MDA-MB-231	**53**
21PT	53
21MT-1	0

B

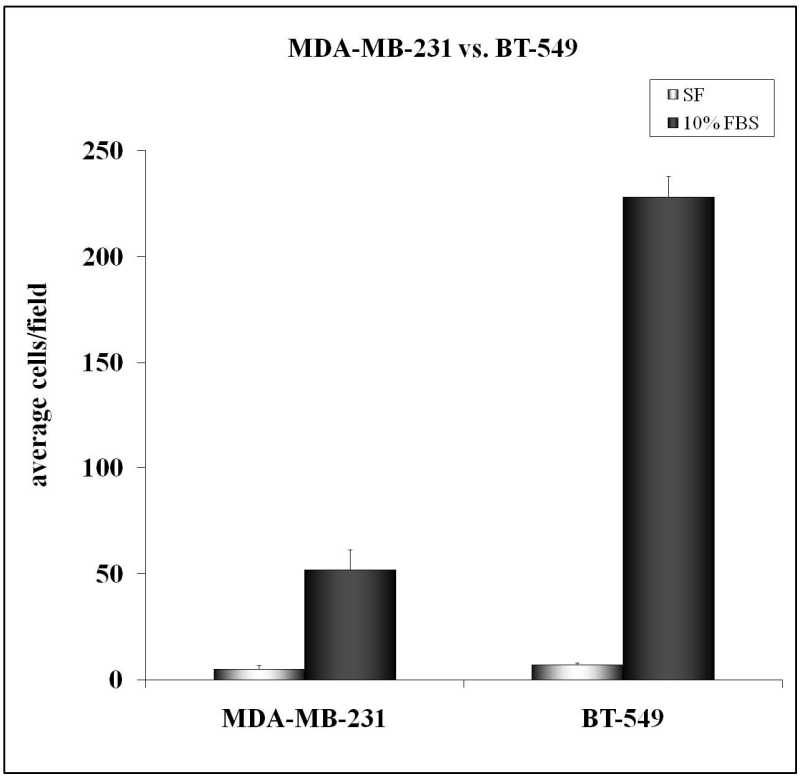
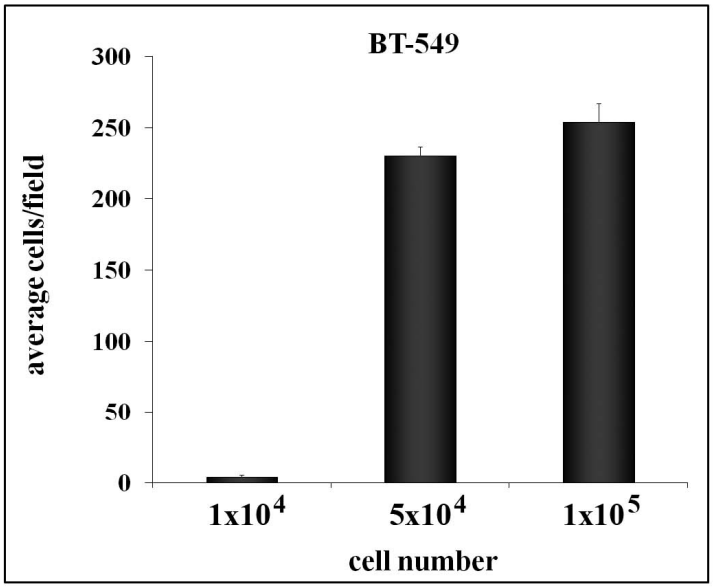


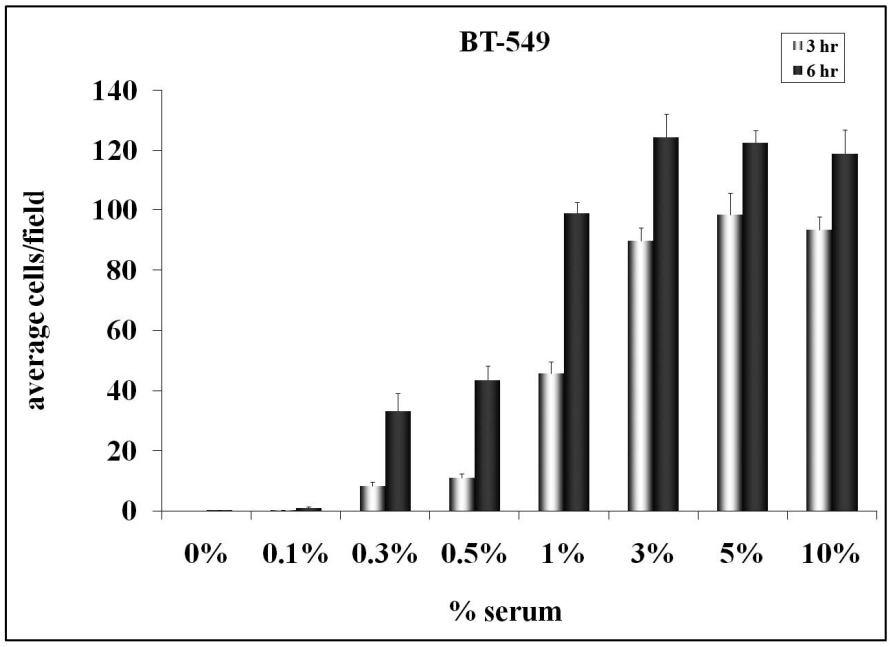
Figure 6: Optimization of BT-549 Migration Conditions

(A) Different numbers of BT-549 cells ($1x10^4$, $5x10^4$, $1x10^5$) were plated in serum-free media in upper chambers of transwell migration plates, with media containing 10% FBS placed in the lower chambers. Cells were allowed to migrate for 6 hours and then fixed, stained, and counted. Results are graphed as average number of migratory cells per field for one experiment. (B) $5x10^4$ BT-549 cells were plated in upper chambers of transwell migration plates with varying concentrations of serum placed in the lower chambers. Cells were allowed to migrate for 3 or 6 hours. Results are graphed as average number of migratory cells per field for one experiment.

A


BT-549
average cells/field
300
250
200
150
100
50
0
1x10^4
5x10^4
1x10^5
cell number

B


BT-549
3 hr
6 hr
average cells/field
140
120
100
80
60
40
20
0
0%
0.1%
0.3%
0.5%
1%
3%
5%
10%
% serum

of cells. Next, using $5x10^4$ cells, we simultaneously tested serum concentration (0-10%) and duration of the assay (3 or 6 hours). BT-549 cells migrated at similar levels to serum concentrations of 3% and greater at both time points (Figure 6B). A substantial difference was seen between time points at 0.3%, 0.5%, and 1% serum levels. However, overall migration to 0.3% and 0.5% serum was low. Therefore, we chose to use $5x10^4$ cells, 1% serum, and 3 hours as our transwell migration conditions for the BT-549 breast cancer cell line. We continued to use $1x10^5$ cells, 10% serum, and 6 hours for the MDA-MB-231 cell line.

Examining Breast Cancer Cell Migration to EGF

Next, we examined whether this migration to serum was chemotactic using a checkerboard assay. In this assay, BT-549 cells were plated in the upper wells of transwell plates in the presence of varying concentrations of serum. Serum concentrations were also varied in the lower chambers in order to create positive serum gradients, in which serum concentrations were higher in the lower chambers, or negative serum gradients in which serum concentrations were higher in the upper chambers. BT-549 cells migrated chemotactically to serum, as seen by an increase in the average number of migratory cells in the presence of a positive serum gradient (Figure 7).

EGF is present in serum and can promote migration of breast cancer cells (Price et al., 1999; Schrecengost et al., 2007; Xue et al., 2006). BT-549 cells overexpress EGFR and STAT5b is a known mediator of EGF signaling in EGFR-overexpressing breast cancer cells (Kloth et al., 2003; Weaver and Silva, 2006). Given this information, we investigated whether EGF could promote migration of BT-549 breast cancer cells. In the absence of serum, EGF placed in the lower chambers did not stimulate BT-549 or MDA- MB-231 migration (*data not shown*).

Figure 7: Breast Cancer Cells Migrate Chemotactically to Serum

BT-549 cells were plated in transwell plates with media containing varying concentrations of serum in both the upper and lower chambers, as indicated. After 3 hours, cells were fixed, stained, and counted as described above. Results are listed as average number of migratory cells per field $\pm$ SE for three independent experiments. Values located above the line indicate migration under a negative serum gradient, with serum concentrations higher in the upper chamber. Values located below the line indicate migration under a positive serum gradient, with serum concentrations higher in the lower chamber. Values in bold, located between the two lines, indicate chemokinetic conditions (serum levels equal in upper and lower chambers).

% Serum in Bottom Chamber \ % Serum in Top Chamber	0	0.5	1.0	3.0
0	0	1 ± 1	6 ± 1	1 ± 1
0.5	32 ± 8	21 ± 4	13 ± 1	15 ± 3
1.0	77 ± 9	32 ± 3	27 ± 7	23 ± 6
3.0	110 ± 7	82 ± 2	56 ± 10	15 ± 2

To test whether serum was necessary to see an effect of EGF on migration, we placed 1% serum in both chambers to create an equal serum gradient, and added varying amounts of EGF to the lower chambers. We observed a small increase in migration to 0.1, 1, or 10 ng/ml EGF compared to no EGF in the presence of an equal serum gradient (Figure 8A). This experiment was only performed once and must be repeated to determine whether the observed increase with EGF is statistically significant. Furthermore, we did not test the effect of EGF with varying serum concentrations. Because substantial levels of migration occurred in the presence of 1% serum, perhaps lowering the concentration of serum would increase the relative difference in migration. Nonetheless, although EGF treatment induced STAT5b tyrosine phosphorylation in BT-549 cells, we never observed serum-induced tyrosine phosphorylation of STAT5b or serum-induced transcriptional activity of a STAT5b-specific luciferase reporter (Figure 8B, *data not shown*). Given these data, we used total serum rather than EGF as the migratory stimulus for future studies.

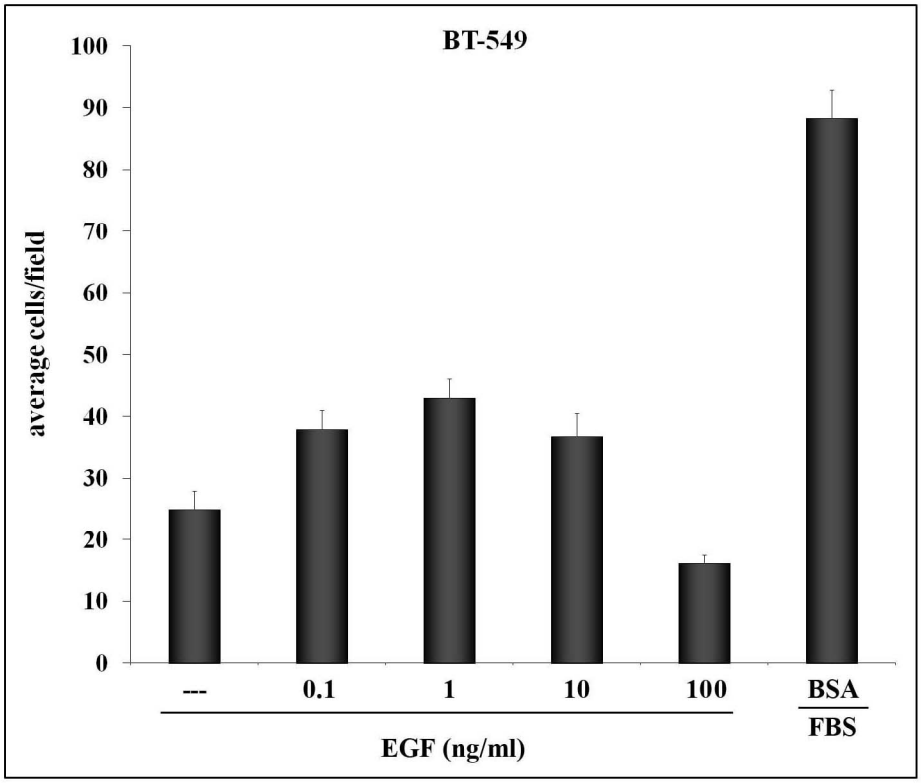
STAT5b Knockdown Inhibits Breast Cancer Cell Migration and Invasion

To determine the importance of STAT5b for breast cancer cell migration, we took an siRNA approach to knockdown STAT5b in BT-549 breast cancer cells. Knockdown of STAT5b to undetectable levels in these cells inhibited their migration to serum by approximately 70% (Figure 9A). To ensure that this effect was not unique to one cell line, we also tested the effect of STAT5b siRNA on migration of MDA-MB-231 cells. In these cells, specific knockdown of STAT5b inhibited migration to serum by approximately 50% (Figure 9B).

Figure 8: Examining Breast Cancer Cell Migration to EGF

(A) BT-549 cells were plated in transwell chambers with media containing 1% FBS in both the upper and lower chambers. Varying concentrations of EGF (0.1, 1, 10, 100 ng/ml) was added to the lower chambers only. As a control, cells were also plated in serum-free media in the upper chamber with media containing 1% FBS in the lower chamber (*far right lane, BSA/FBS*). After 3 hours, cells were fixed, stained, and counted. Results are graphed as average number of migratory cells per field for one independent experiment. (B) BT-549 cells were serum-starved (DMEM + 0.1% BSA) overnight, then treated with media alone (con), 100ng/ml EGF (EGF) or 200ng/ml GH (GH) for 15 minutes at 37^{o}C. Cell were lysed and immunoprecipitated with an antibody specific for STAT5b. Immunoprecipitates were blotted with antibodies against phospho-tyrosine 699 STAT5b (pY699-STAT5b, *top*) and total STAT5b (STAT5b, *bottom*).

A



B

BT-549

IP:STAT5b

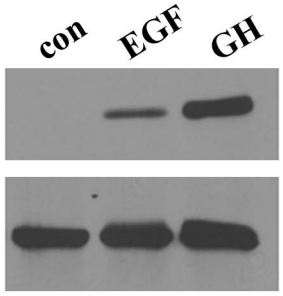
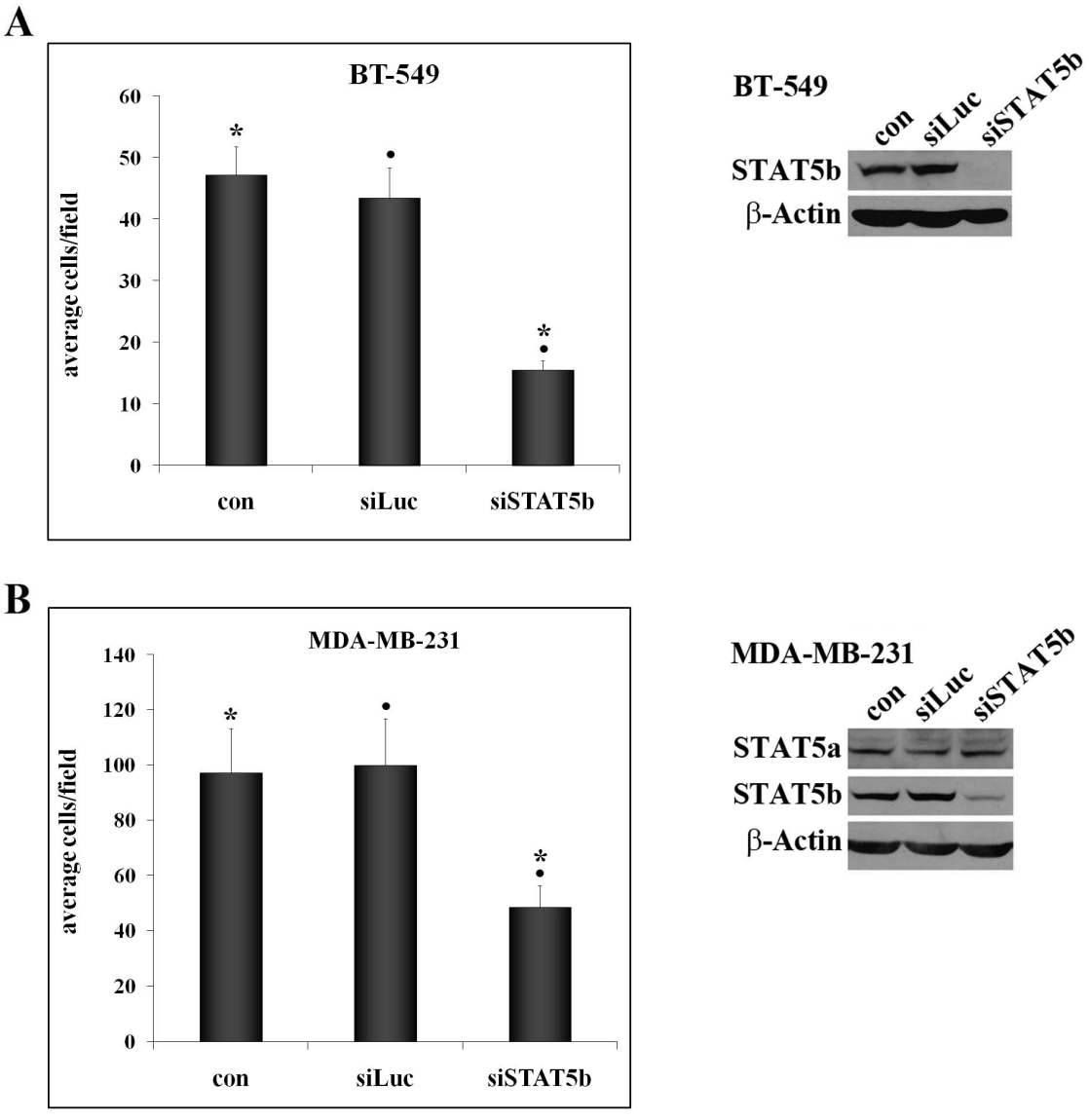


Figure 9: Knockdown of STAT5b Inhibits Breast Cancer Cell Migration

(A) BT-549 breast cancer cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc) or siSTAT5b SMARTpool (siSTAT5b) using Oligofectamine. Seventy-two hours following transfection, cells were plated in serum-free media in upper chambers, and media containing 1% FBS was placed in the lower chamber. After 3 hours the cells were fixed, stained, and the number of migratory cells was counted. Four fields were counted on each of two filters. Results are graphed as the average number of migratory cells per field $\pm$ SE. One-way ANOVA with Tukey's post-test was used to determine statistical significance between the following: con and siSTAT5b *, $p<0.05$; siLuc and siSTAT5b •, $p<0.05$ (n=5). (B) MDA-MB-231 cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc), or siRNA specific to STAT5b (siSTAT5b siGENOME SMARTpool oligonucleotide #3) and migration was measured in the same manner as BT-549 cells except that transwell assays were performed using 10% FBS in the lower chambers for 6 hours. Results are graphed as the average number of migratory cells per field $\pm$ SE. One-way ANOVA with Tukey's post-test was used to determine statistical significance between the following: con and siSTAT5b *, $p<0.05$; siLuc and siSTAT5b •, $p<0.05$ (n=4). (A, B) *Right panels* - Whole cell lysates from siRNA-transfected BT-549 and MDA-MB-231 breast cancer cells were collected seventy-two hours post-transfection and immunoblotted with antibodies specific for STAT5a or STAT5b, and β–actin as a loading control.

** Figure 1A of manuscript*


A
BT-549
average cells/field
60
50
40
30
20
10
0
*
*
con
siLuc
siSTAT5b
BT-549
con
siLuc
siSTAT5b
STAT5b
β-Actin
B
MDA-MB-231
average cells/field
140
120
100
80
60
40
20
0
*
*
con
siLuc
siSTAT5b
MDA-MB-231
con
siLuc
siSTAT5b
STAT5a
STAT5b
β-Actin

Additionally, STAT5b knockdown inhibited invasion of BT-549 breast cancer cells through Matrigel (Figure 10). Given the low number of invasive cells per viewing field (Figure 10A), we also counted the total number of invasive cells on each filter (Figure 10B), and calculated relative invasion compared to control siRNA (Figure 10C). We attempted to verify this effect in MDA-MB-231 cells, but were not able to see significant invasion under control conditions (*data not shown*).

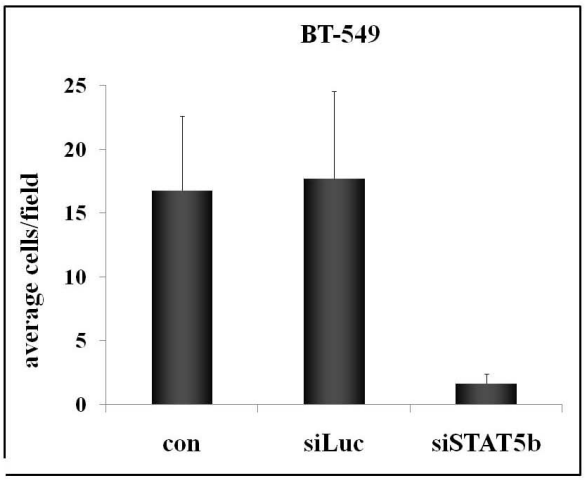
Since BT-549 cells only required low amounts of serum to migrate (Figure 6B), we utilized them to determine whether increasing concentrations of serum could overcome the effect of STAT5b knockdown. As seen in Figure 11A, migration to serum was dose-dependent with 3-10% serum being optimal. Knockdown of STAT5b significantly inhibited migration of BT-549 cells by 60-80% at all serum concentrations. Similar results were obtained in the MDA-MB-231 cells (Figure 11B). Thus, increasing the concentration of serum components did not overcome the effect of STAT5b knockdown on inhibiting migration. However, migration was not completely abrogated upon STAT5b knockdown, and the cells retained some capacity to migrate. These results suggest that there may be both STAT5b-dependent and STAT5b-independent pathways responsible for migration of these cell lines to serum.

Given that STAT5b knockdown inhibited breast cancer cell migration (Figures 9, 11), we next tested whether overexpression of STAT5b could increase migration. Overexpression of STAT5b in non-migratory SKBr3 breast cancer cells did not stimulate migration (*data not shown*). Overexpression of STAT5b in migratory BT-549 cells also had no effect (Figure 12). Since STAT5a overexpression has been shown to suppress migration of BT-20 and T47D breast cancer cells, we also tested the effect of STAT5a expression on breast cancer cell migration.

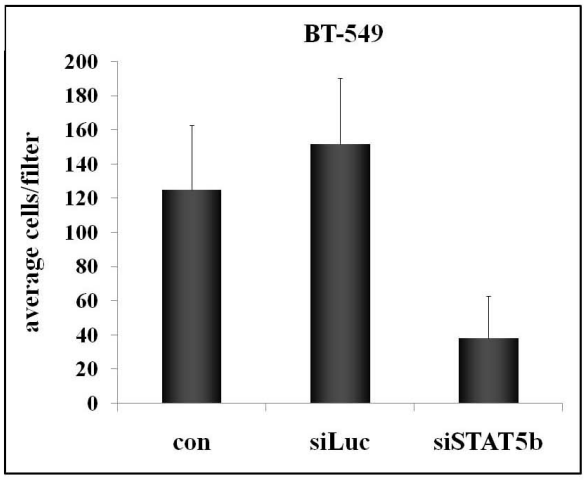
Figure 10: Knockdown of STAT5b Inhibits Breast Cancer Cell Invasion

BT-549 breast cancer cells were transfected with siRNA as described in Figure 9. Forty-eight hours following transfection, cells were plated in serum-free media in transwell invasion chambers, and media containing 10% FBS was placed in the lower chambers. After twenty-four hours non-invasive cells were removed and the remaining cells were fixed, stained, and counted. Four fields on each of two filters or entire filters were counted. Results are graphed as the average number of invasive cells per field (A), per filter (B), or relative invasion (C) (*fold vs. control*) $\pm$ SE. (A) n=2. (B) n=3. (C) One-way ANOVA with Tukey's post-test was used to determine statistical significance between the following: con and siSTAT5b *, $p<0.05$; siLuc and siSTAT5b •, $p<0.05$ (n=3).

A


BT-549
average cells/field
25
20
15
10
5
0
con
siLuc
siSTAT5b

B


BT-549
average cells/filter
200
180
160
140
120
100
80
60
40
20
0
con
siLuc
siSTAT5b

C

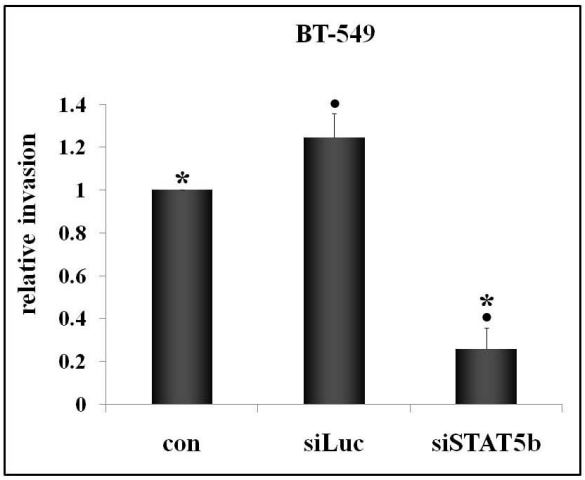
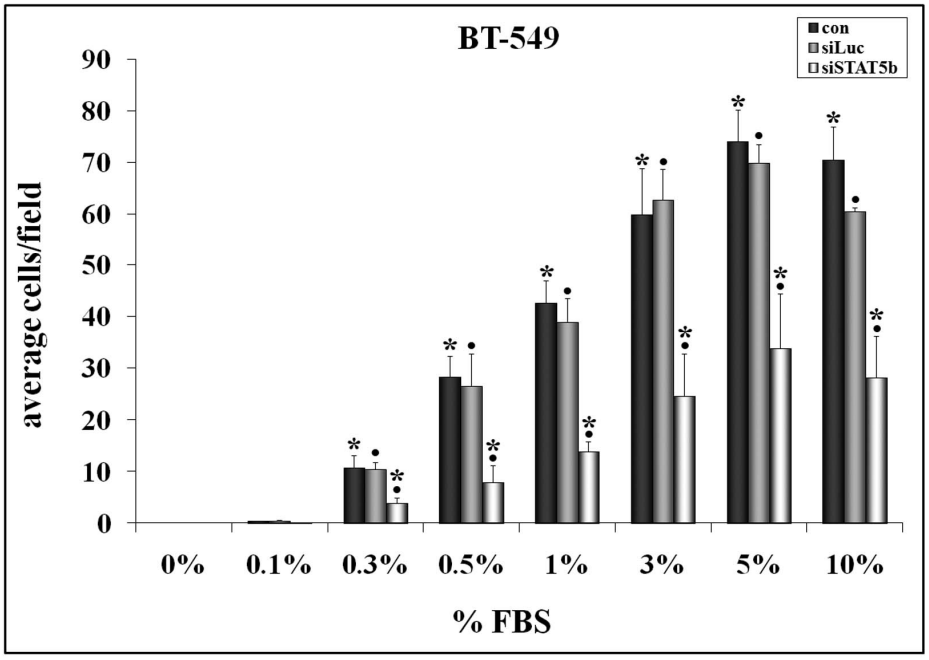

BT-549
relative invasion
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
•
*
•
con
siLuc
siSTAT5b

Figure 11: Knockdown of STAT5b Inhibits Breast Cancer Cell Migration Independent of Serum Concentration

(A,B) BT-549 and MDA-MB-231 breast cancer cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc) or siSTAT5b SMARTpool (siSTAT5b), and transwell migration assays were performed as described in Figure 9. Media containing varying concentrations of FBS (0.1%-10%) was placed in the lower chambers. Four fields were counted on each of two filters. Results are graphed as the average number of migratory cells per field $\pm$ SE. (A) One-way ANOVA with Tukey's post-test was used to determine statistical significance between the following: con and siSTAT5b *, $p<0.05$; siLuc and siSTAT5b •, $p<0.05$ (n=3). (B) n=1.

** Part A is Figure 1B of manuscript*

A


BT-549
con
siLuc
siSTAT5b
average cells/field
90
80
70
60
50
40
30
20
10
0
0%
0.1%
0.3%
0.5%
1%
3%
5%
10%
% FBS

B

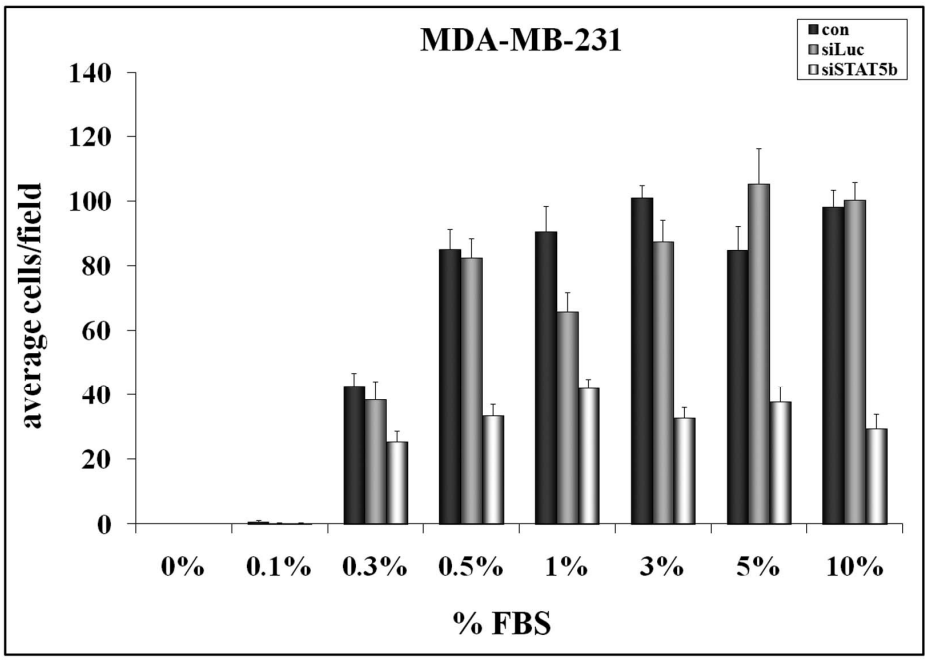
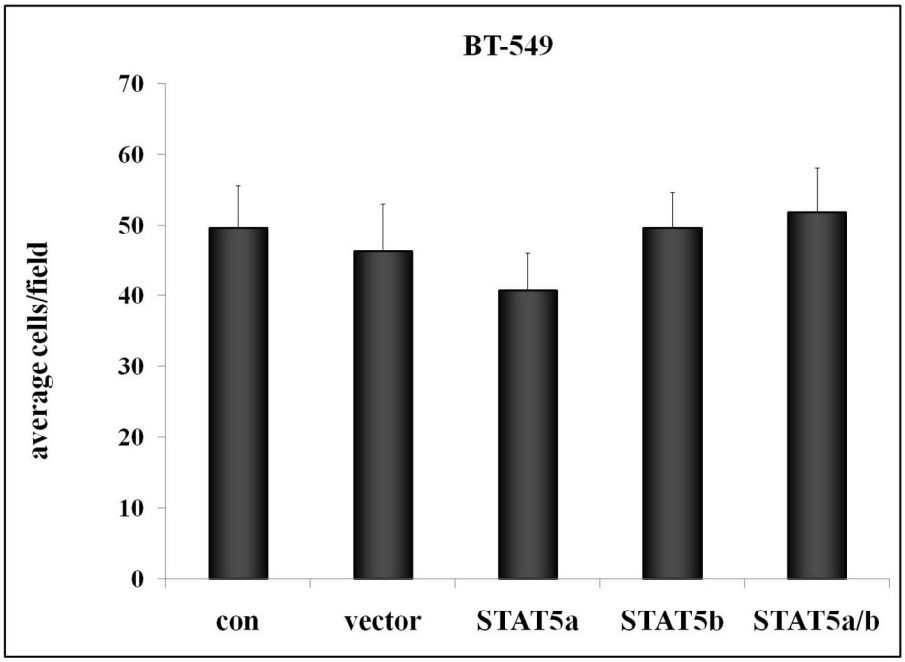

MDA-MB-231
con
siLuc
siSTAT5b
average cells/field
140
120
100
80
60
40
20
0
0%
0.1%
0.3%
0.5%
1%
3%
5%
10%
% FBS

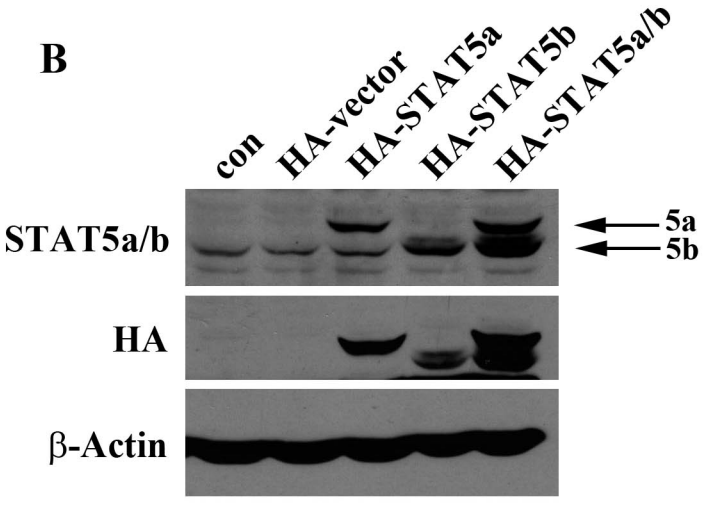
Figure 12: Testing Overexpression of STAT5a or STAT5b on Breast Cancer Cell Migration

(A) BT-549 cells were transfected with HA-vector or HA-tagged STAT5a, STAT5b, or both using Lipofectamine. Forty-eight hours post-transfection, transwell migration assays were performed with 1% FBS for 3 hours as described in Figure 9. Untransfected cells (con) served as an additional control. Four fields were counted on each of two filters. Results are graphed as the average number of migratory cells per field $\pm$ SE. Comparisons made between control and transfected conditions were not statistically significant by one-way ANOVA with Tukey's post-test (n=3). (B) Whole cell lysates from transfected cells were collected forty-eight hours post-transfection and immunoblotted with antibodies specific for STAT5a/b or HA, and β–actin as a loading control.

A


BT-549
average cells/field
70
60
50
40
30
20
10
0
con
vector
STAT5a
STAT5b
STAT5a/b

B


con
HA-vector
HA-STAT5a
HA-STAT5b
HA-STAT5a/b
STAT5a/b
5a
5b
HA
β-Actin

Overexpression of STAT5a neither suppressed nor promoted the migration of BT-549 breast cancer cells (Figure 12). The limitation of these studies is that total populations of cells were counted rather than transfected cells only. Although Western Blots showed significant overexpression of STAT5a and STAT5b (Figure 12B), this may be due to low overexpression of a large number of cells or high overexpression in a small subset of cells. Analysis of transfected cells only, is necessary to confirm this result.

The effect of STAT5b Knockdown on Migration is Not Due to Indirect Effects on Adherence, Proliferation or Survival

It is well established that STAT5b promotes cell cycle progression and survival of breast cancer cells (Fox et al., 2008; Kloth et al., 2003; Riggins et al., 2006; Weaver and Silva, 2006; Yamashita et al., 2003; Yamashita et al., 2004). To determine whether knockdown of STAT5b affected cell cycle, survival, or proliferation under the conditions of our migration experiments, we performed trypan blue assays and flow cytometry on knockdown cells at seventy-two hours post-transfection, the same time point at which migration assays were performed. In both BT-549 and MDA-MB-231 cells, knockdown of STAT5b did not significantly alter the total number of adherent cells or the viability of adherent cells over a six hour time period, which is the maximum length of our transwell migration assays (Table 2). We also examined the distribution of cells in each phase of the cell cycle following STAT5b knockdown. In BT-549 breast cancer cells, knockdown of STAT5b increased the percentage of cells in G1 and decreased the percentage of cells in S phase compared to control siLuc knockdown (Figure 13A). A different effect was seen in MDA-MB-231 breast cancer cells, where knockdown of STAT5b increased the percentage of cells in S phase and decreased the

Table 2: Effect of STAT5b Knockdown on Adherence and Viability

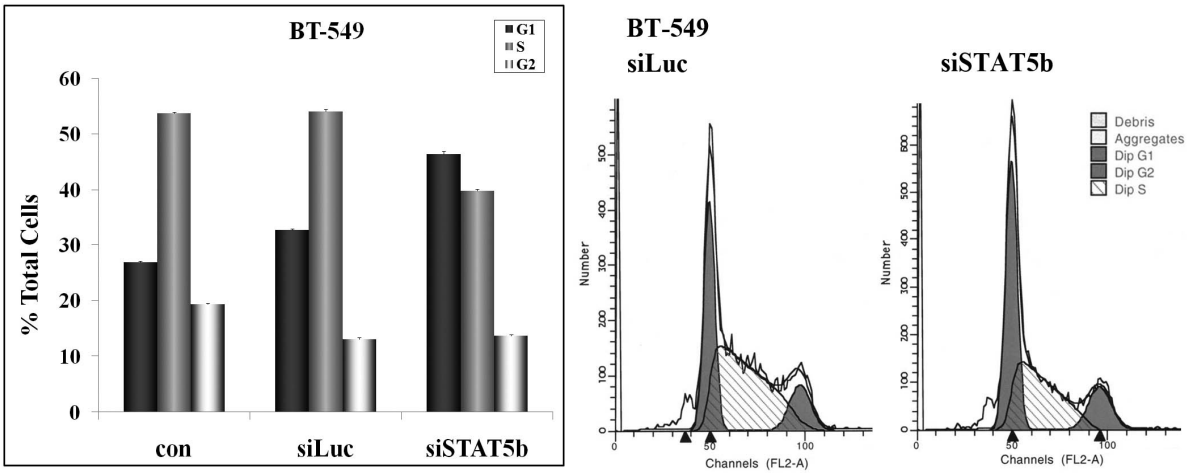
Results listed are for three independent experiments. Comparisons were made between con and siSTAT5b, and siLuc and siSTAT5b. None of these values were statistically significant using one-way ANOVA with Tukey's post-test.

Cell Line	siRNA	Adherent Cells (% ± SE)	Viable Adherent Cells (% ± SE)	Non-viable Adherent Cells (% ± SE)
BT-549	con	68 ± 6	75 ± 11	25 ± 11
	siLuc	59 ± 5	75 ± 5	25 ± 5
	siSTAT5b	58 ± 5	73 ± 8	27 ± 8
MDA-MB-231	con	80 ± 3	87 ± 2	13 ± 2
	siLuc	82 ± 2	92 ± 1	8 ± 1
	siSTAT5b	74 ± 5	84 ± 4	16 ± 4

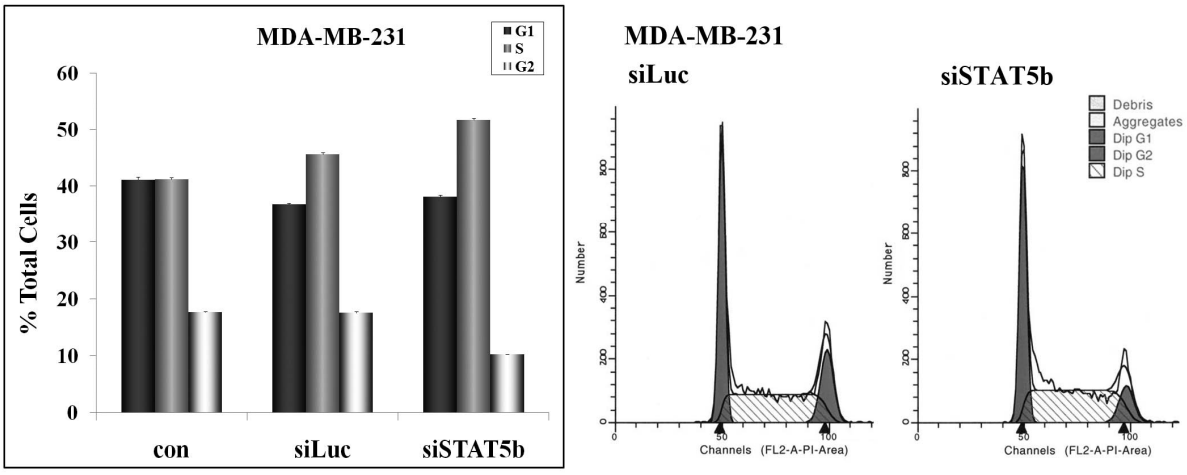
Figure 13: Cell Cycle Distribution Following STAT5b Knockdown

BT-549 (A) and MDA-MB-231 (B) breast cancer cells were transfected with siRNA as described in Figure 9. Seventy-two hours following transfection, cells were collected and fixed in 70% ethanol for 2 hours at 4^{o}C. After fixation, cells were stained with a solution of 40µg/ml (BT-549) or 20µg/ml (MDA-MB-231) propidium iodide in 0.1% Triton X-100 in PBS for one hour at room temperature, and flow cytometry was performed. Results are graphed as the percentage of cells in each phase of the cell cycle (*left*) and represent an average of three triplicates from one experiment. A representative staining profile is shown on the *right*.

A


BT-549
G1
S
G2
% Total Cells
60
50
40
30
20
10
0
con
siLuc
siSTAT5b
BT-549
siLuc
siSTAT5b
Debris
Aggregates
Dip G1
Dip G2
Dip S
Number
Channels (FL2-A)

B


MDA-MB-231
G1
S
G2
% Total Cells
60
50
40
30
20
10
0
con
siLuc
siSTAT5b
MDA-MB-231
siLuc
siSTAT5b
Debris
Aggregates
Dip G1
Dip G2
Dip S
Number
Channels (FL2-A-PI-Area)

percentage of cells in G2 (Figure 13B). These experiments were done simultaneously with transwell migration assays in which knockdown of STAT5b inhibited migration in both cell lines (Figure 9).

Knockdown of STAT1, but Not STAT5a or STAT3, Inhibits Breast Cancer Cell Migration

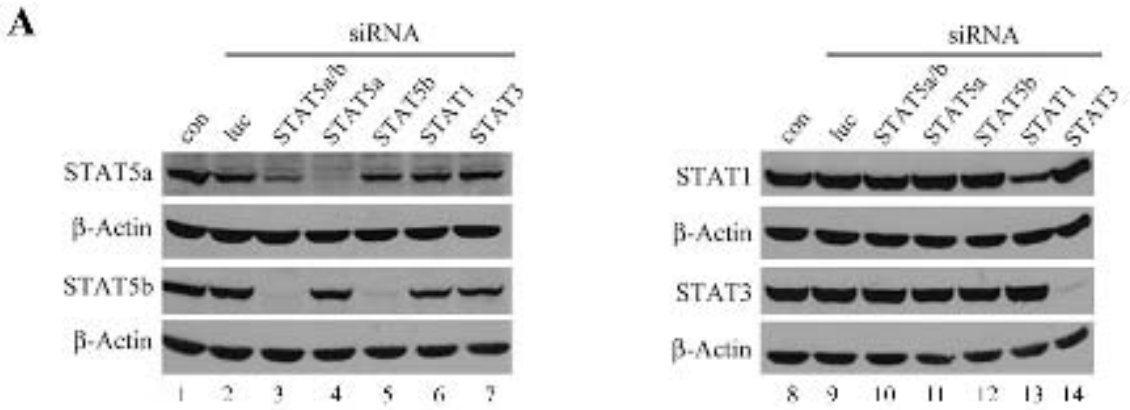
STAT5a and STAT5b are highly homologous and are both activated in breast cancer, and previous work has shown that Prl-induced STAT5a activation suppresses migration of BT-20 and T-47D breast cancer cells (Sultan et al., 2005). Thus, we investigated the role of STAT5a, and other related STATs, STAT1 and STAT3, on migration in our model system. We chose to use the MDA-MB-231 breast cancer cell line because they are highly migratory and express all four STAT proteins. The siSTAT5b SMARTpool siRNA (Dharmacon) used in the BT-549 cell line, while efficient at knocking down STAT5b, also knocked down STAT5a by approximately 60% in MDA-MB-231 cells (Figure 14A, *lane 3*). To specifically target STAT5b in the MDA-MB-231 cells, we used one of the individual oligonucleotides from the SMARTpool, which had no effect on STAT5a levels (Figure 14A, *lane 5*). This single oligonucleotide had been used in previous knockdown experiments in the MDA-MB-231 cell line (Figure 9). The total SMARTpool was utilized to determine the effect of dual knockdown of STAT5a and STAT5b in our MDA-MB-231 model system. It is of note that the BT-549 cell line does not express STAT5a (*data not shown*).

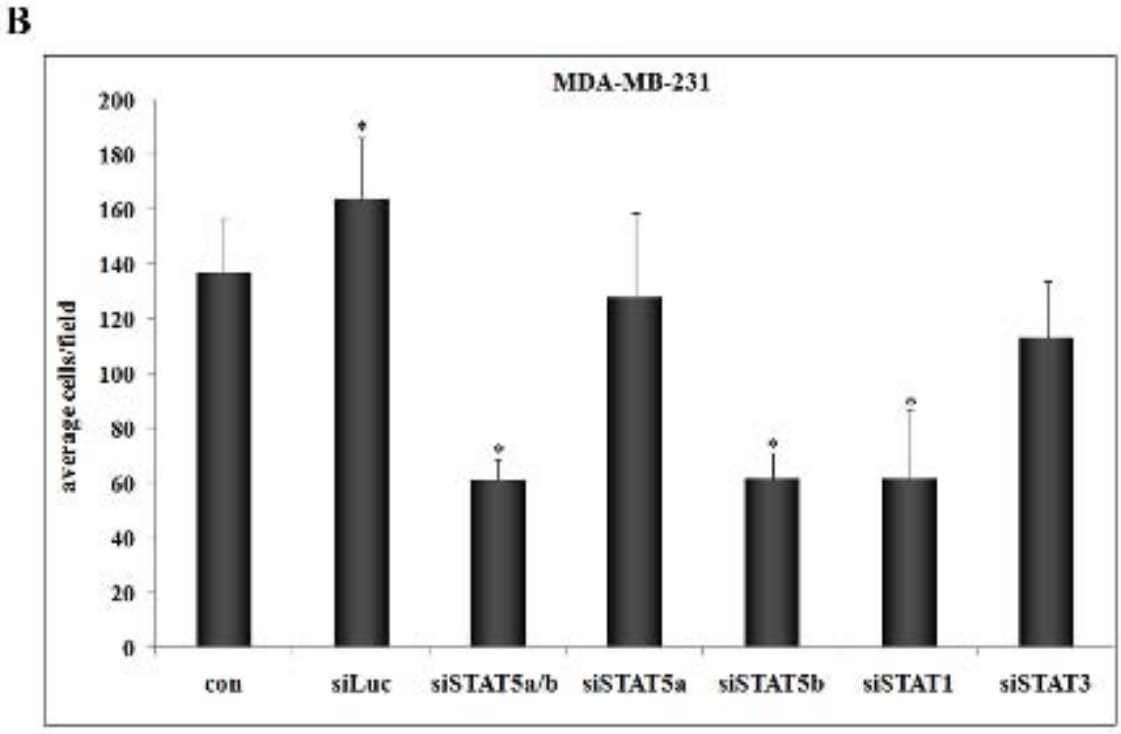
As seen previously, knockdown of STAT5b inhibited migration of MDA-MB-231 cells by greater than 50%, but knockdown of STAT5a had no effect on migration (Figure 14B). Furthermore, knockdown of STAT5a in combination with STAT5b did not enhance the inhibition of migration due to knockdown of STAT5b alone. Similar to STAT5a, knockdown of

Figure 14: Knockdown of STAT5b or STAT1, but Not STAT5a or STAT3 Inhibits Breast Cancer Cell Migration

(A) MDA-MB-231 cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc), or siRNA specific to STAT5a/b (siSTAT5b siGENOME SMARTpool), STAT5a (siSTAT5a custom oligonucleotide), STAT5b (siSTAT5b siGENOME SMARTpool oligonucleotide #3), STAT1 (siSTAT1 siGENOME SMARTpool), or STAT3 (siSTAT3 siGENOME SMARTpool). Whole cell lysates were collected seventy-two hours post-transfection and immunoblotted with antibodies specific for STAT5a, STAT5b, STAT1, or STAT3, and β–actin as a loading control. (B) Seventy-two hours following transfection, transwell migration assays were performed for 6 hours as described in Figure 9. Results are graphed as average number of migratory cells per field $\pm$ SE. One-way ANOVA with Tukey's post-test was used to determine statistical significance between the conditions, with the following being statistically significant: siLuc and siSTAT5a/b *, $p<0.05$; siLuc and siSTAT5b *, $p<0.05$; siLuc and siSTAT1 *, $p<0.05$ ($n\geq4$). Comparisons of con and siLuc, siLuc and siSTAT5a, and siLuc and siSTAT3 were not statistically significant.

** Figure 1C of manuscript (excluding STAT1 and STAT3 siRNA)*


A
siRNA
con
luc
STAT5a/b
STAT5a
STAT5b
STAT1
STAT3
STAT5a
β-Actin
STAT5b
β-Actin
1 2 3 4 5 6 7
siRNA
con
luc
STAT5a/b
STAT5a
STAT5b
STAT1
STAT3
STAT1
β-Actin
STAT3
β-Actin
8 9 10 11 12 13 14


B
MDA-MB-231
average cells/field
200
180
160
140
120
100
80
60
40
20
0
con
siLuc
siSTAT5a/b
siSTAT5a
siSTAT5b
siSTAT1
siSTAT3

STAT3 did not affect migration of MDA-MB-231 cells. In contrast, knockdown of STAT1 significantly inhibited migration, to levels similar to that seen with STAT5b knockdown (55%). One independent experiment examining the effects of STAT1 and STAT3 knockdown was performed in BT-549 cells (*data not shown*). Similar to MDA-MB-231 cells, knockdown of STAT1 in BT-549 cells significantly inhibited migration. Interestingly, knockdown of STAT3 in BT-549 cells also significantly inhibited migration, suggesting a differential role for STAT3 in migration of these two cell lines to serum. Additional experiments in the BT-549 cell line must be performed to confirm these results.

B. Identify the component(s) of serum responsible for breast cancer cell migration to serum and whether STAT5b is necessary for migration to this serum component

STAT5b Knockdown Inhibits β1 Integrin-Mediated Migration to FN

As a means of gaining insight into those migratory signaling pathways to which STAT5b contributes, we set out to determine the serum component responsible for migration of breast cancer cells. BT-549 migration to serum is chemotactic, meaning that the cells migrate to a chemical gradient (Figure 7). The term chemokine is used to describe the soluble chemical stimuli that induce chemotaxis. Chemokines bind to G-protein linked membrane receptors and transduce signals into the cell stimulating migration. CXCR4 and CCR7 are the two major chemokine receptors expressed on breast cancer cells (Muller et al., 2001). Therefore, we examined migration of BT-549 cells to ligands for these receptors, stromal cell-derived factor-1α or β (SDF-1α,β) and CCL21, respectively. Despite previously published work, we did not

observe migration of BT-549 or MDA-MB-231 cells to these chemokines (Figure 15B) (Muller et al., 2001).

We next examined migration to lysophosphatidic acid (LPA), a major component of serum known to stimulate fibroblast migration (Moolenaar, 2002). Neither cell line migrated to LPA (Figure 15C). We also tested the serum components insulin and insulin-like growth factor, but observed no migration (*data not shown*). To determine if hormones or small peptides were involved, we charcoal-stripped serum prior to migration assays. MDA-MB-231 cells migrated similarly to total and charcoal-stripped serum (Figure 15C, *right*). BT-549 cells appeared to migrate less efficiently to charcoal-stripped serum compared to total serum (Figure 15C, *left*), suggesting that hormones and growth factors may be required for optimal migration to serum. Additional experiments are required to determine if this difference is statistically significant. Nevertheless, both cell lines migrated well to charcoal-stripped serum, indicating that small peptides are not the main components of serum stimulating migration.

Having ruled out chemokines, hormones, and growth factors, we assessed migration of cells to extracellular matrix components known to be present in serum, specifically fibronectin (FN) and vitronectin (VN). Both BT-549 and MDA-MB-231 cells migrated to FN, whereas only BT-549 cells migrated significantly to VN (Figure 16A). Therefore, in order to compare effects on both cell lines, we used fibronectin as the migratory stimulus. Cells attach to fibronectin and mediate signaling through integrin receptors comprised of beta 1 (β1) integrin in combination with various alpha integrins. To confirm that fibronectin was indeed a major serum component stimulating migration, we pre-treated cells with a β1 integrin blocking antibody and measured migration to serum. Inhibition of β1 integrin receptor inhibited migration of both cell lines to serum by approximately 50% (Figure 16B). Non-specific mouse IgG had no effect on migration

Figure 15: Testing Migration of BT-549 Cells to Chemotactic Stimuli

(A) BT-549 and MDA-MB-231 whole cell lysates were immunoblotted with antibodies specific for CXCR4 and β–actin as a loading control. (B) Transwell migration assays were performed with BT-549 cells for 3 hours with serum-free media alone (no-serum) or containing 100ng/ml SDF-1α, SDF-1β, or CCL21 in the lower chambers. 1% FBS in the lower chamber served as a positive control. Results are graphed as average number of migratory cells per field for one independent experiment. (C) Transwell migration assays were performed with BT-549 or MDA-MB-231 cells for 3 or 6 hours, respectively. Lower chambers contained serum-free media (no serum), FBS (serum), charcoal-stripped newborn calf serum (cs-serum), or 2μM lysophosphatidic acid (LPA). 1% serum was used for BT-549 cells and 10% was used for MDA-MB-231 cells. Results are graphed as average number of migratory cells per field for one independent experiment.

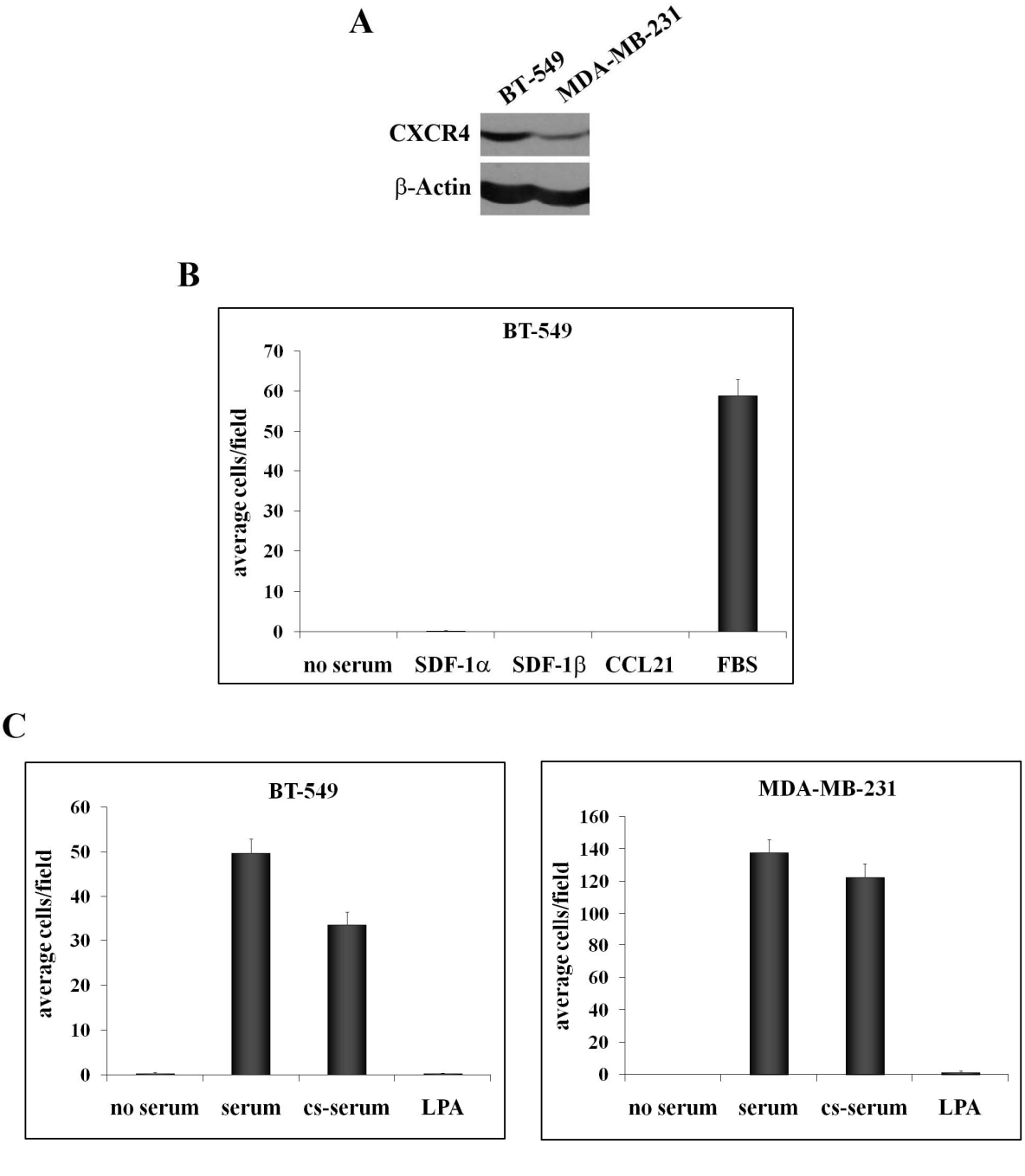
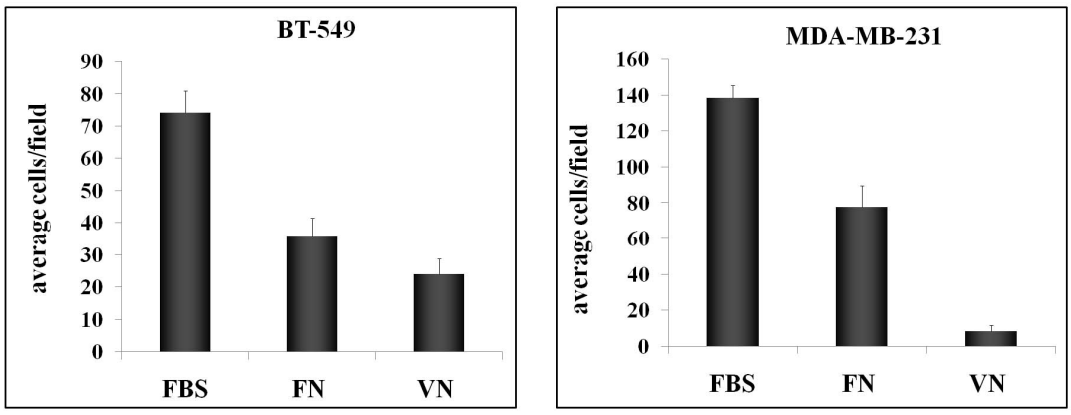

A
BT-549
MDA-MB-231
CXCR4
β-Actin
B
BT-549
average cells/field
70
60
50
40
30
20
10
0
no serum
SDF-1α
SDF-1β
CCL21
FBS
C
BT-549
average cells/field
60
50
40
30
20
10
0
no serum
serum
cs-serum
LPA
MDA-MB-231
average cells/field
160
140
120
100
80
60
40
20
0
no serum
serum
cs-serum
LPA

Figure 16: STAT5b Knockdown Inhibits β1 Integrin-Mediated Migration to Fibronectin

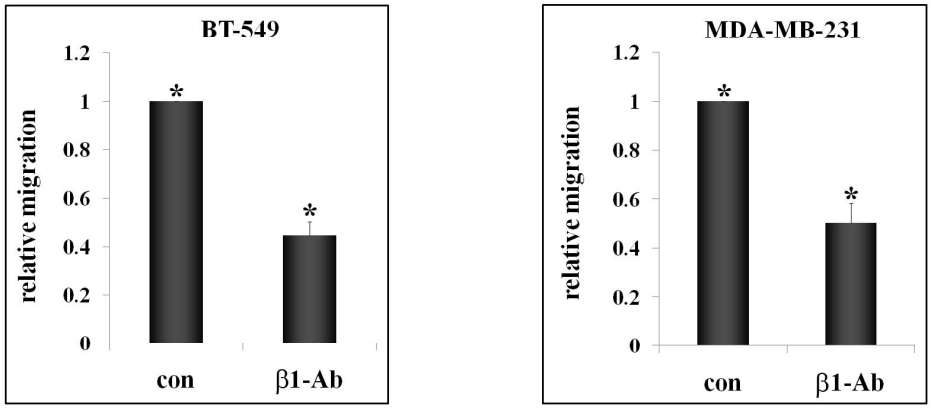
(A) The undersides of transwell filters were coated with 10μg/ml FN or VN overnight at 4°C. BT-549 and MDA-MB-231 cells were placed in serum-free media in upper chambers. Lower chambers contained serum-free media for FN and VN coated filters and 1% FBS (BT-549) or 10% FBS (MDA-MB-231) media for FBS controls. Migration was allowed to proceed for 3 hours (BT-549) or 6 hours (MDA-MB-231). Results are graphed as average number of migratory cells per field (n=3). (B) BT-549 and MDA-MB-231 cells were pre-treated for 1 hour at 37°C with 10μg/ml β1 integrin blocking antibody or DMSO control (con), and treatment was continued for the duration of the assay. Transwell assays were performed with 1% (BT-549) or 10% (MDA-MB-231) FBS in the lower chambers for 3 or 6 hours, respectively. Results are graphed as relative migration (vs. con) $\pm$ SE. Student's *t* test was used to determine statistical significance between the following: BT-549, con and β1 *, $p<0.01$ (n=6); MDA-MB-231, con and β1 *, $p<0.01$ (n=4). (C) The undersides of transwell filters were coated with 3μg/ml FN overnight at 4°C. Transwell migration assays were performed with siRNA transfected cells as described in part A. Results are graphed as relative migration (vs. siLuc) $\pm$ SE. One-way ANOVA with Tukey's post-test was used to determine statistical significance between the following: BT-549, siLuc and siSTAT5b (FBS) *, $p<0.05$; siLuc and siSTAT5b (FN) •, $p<0.05$ (n=3). MDA-MB-231: siLuc and siSTAT5b (FBS) *, $p<0.05$; siLuc and siSTAT5b (FN) •, $p<0.05$ (n=3).

** Figure 3 of manuscript*

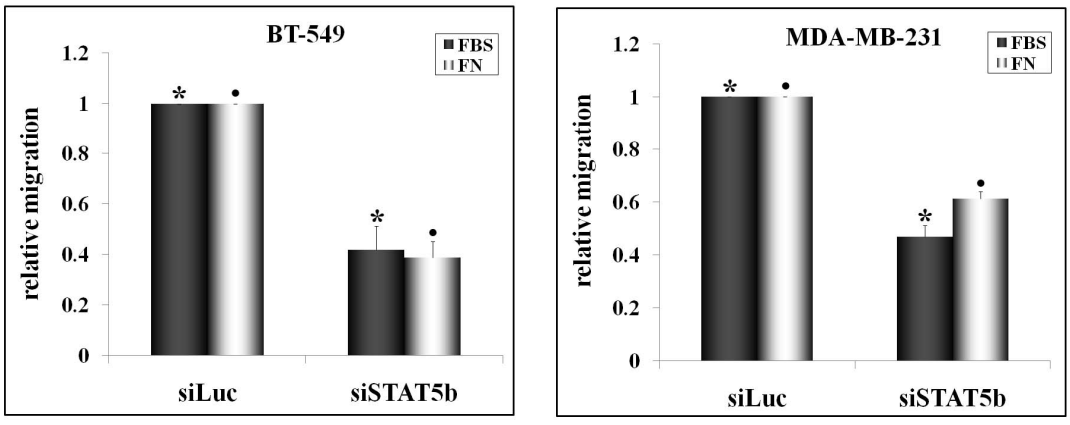
A


BT-549
average cells/field
90
80
70
60
50
40
30
20
10
0
FBS
FN
VN
MDA-MB-231
average cells/field
160
140
120
100
80
60
40
20
0
FBS
FN
VN

B


BT-549
relative migration
1.2
1
0.8
0.6
0.4
0.2
0
*
*
con
β1-Ab
MDA-MB-231
relative migration
1.2
1
0.8
0.6
0.4
0.2
0
*
*
con
β1-Ab

C


BT-549
FBS
FN
relative migration
1.2
1
0.8
0.6
0.4
0.2
0
*
•
*
•
siLuc
siSTAT5b
MDA-MB-231
FBS
FN
relative migration
1.2
1
0.8
0.6
0.4
0.2
0
*
•
*
•
siLuc
siSTAT5b

(*data not shown*). Fibronectin dose curves were performed, and it was found that optimal migration of both BT-549 and MDA-MB-231 cell lines occurred at 2-3μg/ml FN (*data not shown*). This is consistent with a study by *Hayman, et al.*, which measured the level of FN in fetal bovine serum to be 25μg/ml, or 2.5μg/ml in 10% FBS/DMEM (Hayman and Ruoslahti, 1979).

To determine if STAT5b is important in β1 integrin-mediated migration, we examined the effect of STAT5b knockdown on migration to FN. Knockdown of STAT5b inhibited migration of both cell lines to FN, to the same extent that knockdown inhibited migration to serum, approximately 40-60% (Figure 16C). Taken together, these results demonstrate that STAT5b is integral for maximal β1 integrin-mediated migration of breast cancer cells to FN.

Task 2: Elucidate the mechanism by which STAT5b promotes breast cancer cell migration

A. Establish whether STAT5b transcriptional activity is necessary for promoting migration

Effect of Actinomycin D and a Small Molecule Inhibitor Targeted to the SH2 Domain of STAT5b on Migration

We have previously shown that knockdown of STAT5b inhibits transcription from the STAT5a/b-specific Spi2.1 luciferase reporter and STAT5b transcriptional activity can be restored by co-transfection of siRNA-immune wild-type STAT5b, but not the Y699F-STAT5b mutant, confirming that Y699F is transcriptionally inactive (Fox et al., 2008). Additionally, dn-STAT5b lacks the C-terminal TAD, so it cannot recruit transcriptional cofactors to regulate gene transcription. To further confirm that active STAT5b-mediated transcription is not required for promoting breast cancer cell migration, we tested the ability of wild-type STAT5b to rescue in the presence of the transcriptional inhibitor actinomycin D. Initially, MDA-MB-231 cells were

tested because the rescue studies were performed in this cell line, but actinomycin D treatment dramatically inhibited MDA-MB-231 migration, indicating that active transcription is vital for migration of these cells (Figure 17A). We next tested the BT-549 cell line, and actinomycin D treatment did not affect migration of these cells. Therefore, we used the BT-549 cells for these studies. As seen previously, knockdown of STAT5b inhibited migration of BT-549 cells to serum, and re-introduction of wt-STAT5b rescued migration (Figure 17B). Importantly, in the presence of actinomycin D, wt-STAT5b was still able to rescue migration, suggesting that active STAT5b-mediated transcription is not required for migration.

To further explore the necessity of a functional SH2 domain, we pre-treated cells with a small molecule inhibitor targeting the STAT5b SH2 domain. The inhibitor was identified by screening for compounds that disrupted the association between STAT5b and a tyrosine phosphorylated peptide derived from the erythropoietin (EPO) receptor (Muller et al., 2008). Fluorescence polarization assays were used to measure binding of recombinant STAT5b protein to a phosphorylated peptide of the EPO receptor. From these assays, *Muller, et al.,* calculated an IC_{50} of 47$\pm$17μM for this inhibitor (Muller et al., 2008). In these assays, this inhibitor was specific for STAT5b over STAT1, STAT3, and Lck kinase at concentrations less than 400μM. In lymphoma cells, significant inhibition of IFNα-induced STAT5a/b tyrosine phosphorylation required slightly higher concentrations (100-200μM), as did inhibition of DNA binding (200-400μM) (Muller et al., 2008).

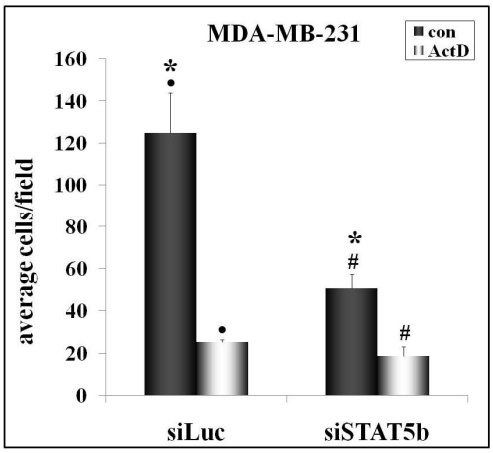
In transwell assays, treatment with this inhibitor decreased migration of both BT-549 and MDA-MB-231 cells at concentrations of 100μM and greater, with almost complete inhibition at 400μM (Figure 17C). Importantly, no effect was seen at 50μM, the *in vitro* IC_{50} of this inhibitor.

Although these data are of potential interest, the *in vivo* efficacy and specificity of this inhibitor in BT-549 and MDA-MB-231 cells must be determined before any conclusions can be made.

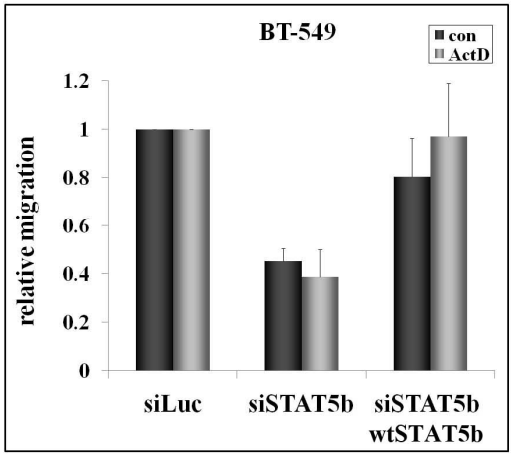
Figure 17: Effect of Actinomycin D and a Small Molecule Inhibitor Targeted to the SH2 Domain of STAT5b on Migration

(A) MDA-MB-231 cells were transfected with siLuc or siSTAT5b siRNA. Seventy-two hours post-transfection cells were pre-treated for 1 hour with DMSO (con) or 10μg/ml actinomycin D (ActD), and treatment maintained for the duration of the assay. Transwell migration assays were performed with 10% FBS placed in lower chambers only, for 6 hours. Results are graphed as average number of migratory cells per field $\pm$ SE. One-way ANOVA with Tukey's post-test was used to determine statistical significance between the following: siLuc con and ActD •, $p<0.05$; siSTAT5b con and ActD #, $p<0.05$; siLuc and siSTAT5b con *, $p<0.05$ (n=3). (B) BT-549 cells were transfected with siLuc, siSTAT5b, or siSTAT5b + wt-STAT5b immune to knockdown. Seventy-two hours post-transfection cells were pre-treated and treated as in part A. Transwell migration assays were performed with 1% FBS in lower chambers, for 3 hours. Results are graphed as relative migration (vs. siLuc) $\pm$ SE (n=2). (C) BT-549 and MDA-MB-231 cells were pre-treated for 1 hour with DMSO (con; 0μM) or indicated concentrations of STAT5b SH2 inhibitor, and treatment was maintained for the duration of the transwell assay (3-6 hours). Results are graphed as relative migration (vs. untreated) BT-549 (n=1); MDA-MB-231 (n=3).

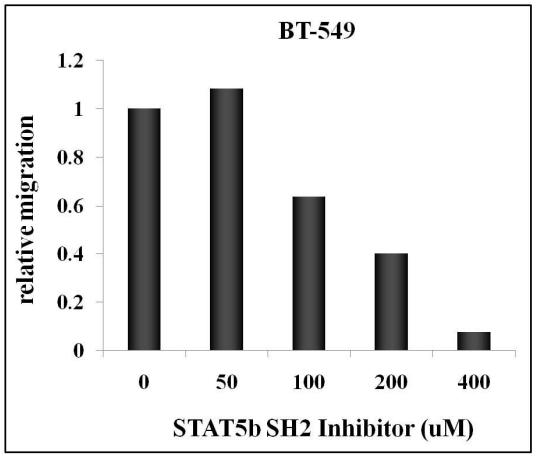
A

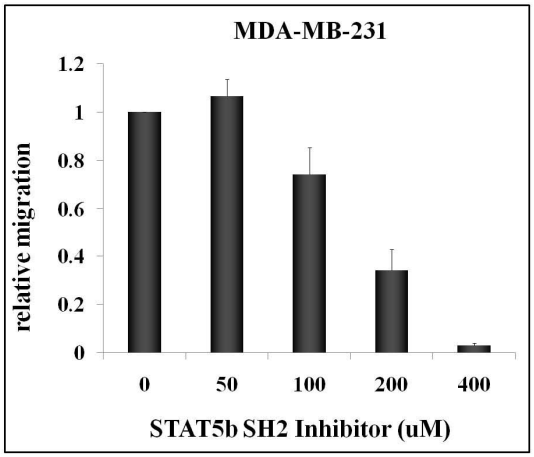

MDA-MB-231
con
ActD
average cells/field
160
140
120
100
80
60
40
20
0
*
•
*
#
•
#
siLuc
siSTAT5b

B


BT-549
con
ActD
relative migration
1.2
1
0.8
0.6
0.4
0.2
0
siLuc
siSTAT5b
siSTAT5b
wtSTAT5b

C


BT-549
relative migration
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
200
400
STAT5b SH2 Inhibitor (uM)


MDA-MB-231
relative migration
1.2
1
0.8
0.6
0.4
0.2
0
0
50
100
200
400
STAT5b SH2 Inhibitor (uM)

B. Identify which region(s) or function(s) of STAT5b is necessary for promoting migration

Expression of Wild-type, Y699F-, or Dominant-negative STAT5b Rescues Migration, but Expression of R618K- or S731A-STAT5b Does Not

First, to eliminate the possibility of off-target effects of STAT5b knockdown, we performed knockdown-rescue experiments. MDA-MB-231 cells were simultaneously transfected with STAT5b-specific siRNA and hemagglutinin (HA)-tagged wild-type STAT5b. Rescue constructs were rendered immune to knockdown by introducing four silent point mutations in the siRNA target sequence. In these experiments, transfection efficiency of siRNA was close to 100%, whereas transfection efficiency of rescue constructs was approximately 65-75% (*data not shown*). Consistent with previous experiments, knockdown of STAT5b inhibited migration of MDA-MB-231 cells by approximately 60%. Re-introduction of wild-type STAT5b restored migration to approximately 76% of control levels (Figure 18), confirming that the inhibition of migration upon STAT5b knockdown is due to a direct effect of STAT5b on migratory pathways.

Additional knockdown-rescue experiments were performed to identify the functional domains required for rescue of the siRNA phenotype. We introduced five mutant forms of STAT5b: (1) Y699F-STAT5b, which cannot be phosphorylated on its conserved tyrosine, Y699; (2) dn-STAT5b (dn-ΔTAD), a C-terminal truncation mutant lacking the transactivation domain (TAD) but retaining Y699; (3) R618K-STAT5b, a SH2 domain mutant containing a mutation in the arginine residue required for binding phosphorylated tyrosine residues; (4) S731A, which cannot be phosphorylated on S731 in the TAD; and (5) 1-144-STAT5b consisting of only the N-terminus. Locations of the indicated domains and residues are shown in Figure 18A. We achieved approximately equal expression of rescue constructs, with the exception of S731A

Figure 18: Expression of Wild-type, Y699F-, or Dominant-negative STAT5b Rescues Migration, but Expression of R618K- or S731A-STAT5b Does Not

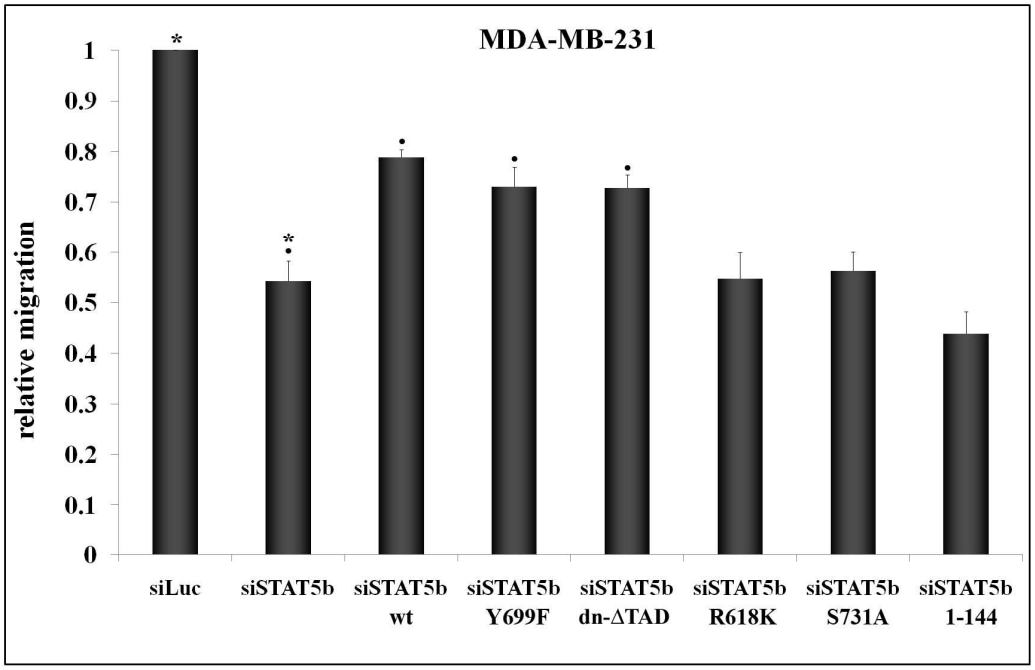
(A) Domain structure of the STAT5b protein depicting the amino terminus (N-term), DNA-binding domain (DBD), Src homology 2 domain (SH2), transactivation domain (TAD), and location of the conserved tyrosine (Y699), arginine (R618), and serine (S731) residues. (B) MDA-MB-231 cells were transfected with control siRNA to luciferase (siLuc) or siRNA specific to STAT5b alone or in the presence of HA-tagged wild-type (wt), Y699F, dominant-negative (dn-ΔTAD), R618K, S731A, or myc-tagged N-terminal (1-144) STAT5b constructs engineered to be immune to siRNA knockdown. Seventy-two hours following transfection, transwell migration assays were performed for six hours to 10% FBS. Results are graphed as relative migration (vs. siLuc) $\pm$ SE. One-way ANOVA analysis with Tukey's post-test was used to determine statistical significance between all constructs. siLuc and siSTAT5b *, $p<0.05$; siSTAT5b and wt, Y699F, or dn •, $p<0.05$ ($n\geq4$). (C) Whole cell lysates from transfected cells were immunoblotted with antibodies specific for STAT5b, HA, myc, or β–actin as a loading control.

** Figure 2 of manuscript (excluding S731A and 1-144 rescue)*

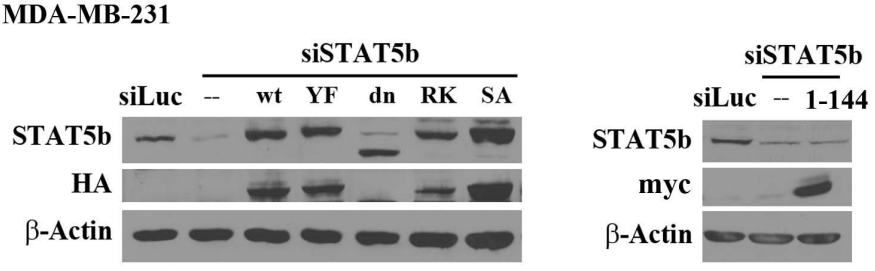
A


STAT5b
R618
Y699
S731
N-term
DBD
SH2
TAD

B


MDA-MB-231
relative migration
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
siLuc
siSTAT5b
siSTAT5b wt
siSTAT5b Y699F
siSTAT5b dn-ΔTAD
siSTAT5b R618K
siSTAT5b S731A
siSTAT5b 1-144

C


MDA-MB-231
siSTAT5b
siLuc
--
wt
YF
dn
RK
SA
STAT5b
HA
β-Actin
siSTAT5b
siLuc
--
1-144
STAT5b
myc
β-Actin

mutant which was expressed at slightly higher levels (Figure 18C). Endogenous STAT5b knockdown was not affected by simultaneous transfection of siRNA and rescue constructs. The C-terminal truncated dn-STAT5b has a lower molecular weight than endogenous STAT5b, allowing us to differentiate endogenous and transfected STAT5b on Western Blots. Endogenous STAT5b is almost completely knocked down regardless of co-transfection (Figure 18C, *siSTAT5b vs. siSTAT5b + dn-ΔTAD*).

In transwell migration assays, re-introduction of Y699F- or dn-STAT5b rescued migration to the same levels as wild-type STAT5b (Figure 18B). In contrast, R618K-, S731A, and 1-144-STAT5b could not rescue the defect in migration due to loss of STAT5b. Taken together, these data indicate that Y699 phosphorylation and subsequent STAT5b-mediated transcription is not necessary for regulating migration, but phosphorylation of S731 is important. Moreover, the SH2 domain has an integral role, separate from that of active dimer formation, in promoting migration.

Exploring Homodimer and Heterodimer formation

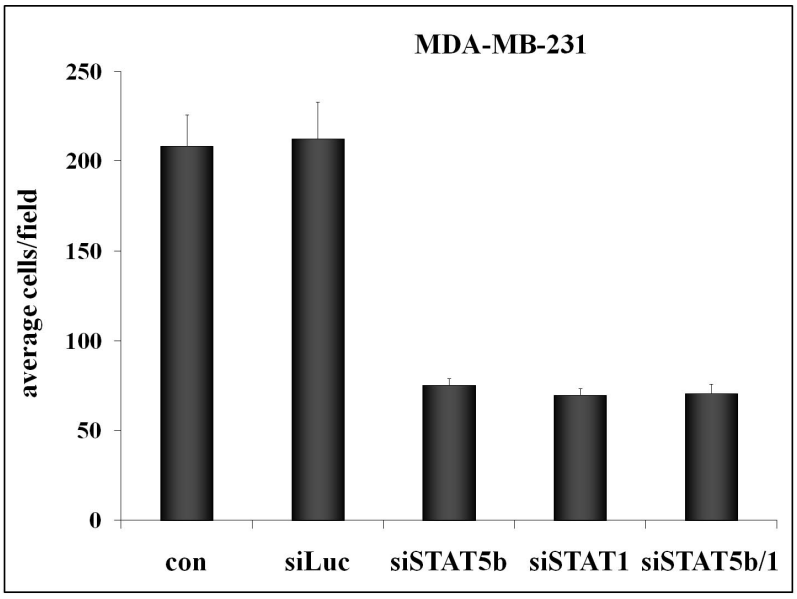
Both phosphorylation of Y699 and an intact SH2 domain are required for active dimer formation of STAT proteins. The finding that Y699F-STAT5b rescued migration as efficiently as wt-STAT5b (Figure 18) suggested that active dimer formation is not necessary for promoting migration, but did not rule out the possibility of signaling through inactive dimers. In rescue studies, endogenous STAT5b was almost completely eliminated through siRNA knockdown, leaving very little endogenous STAT5b for transfected STAT5b to interact with. However, other STAT proteins may heterodimerize with STAT5b, including STAT1. Since knockdown of STAT1 inhibited migration of MDA-MB-231 cells to similar levels as

knockdown of STAT5b (Figures 14, 19), we examined the possibility of association between STAT5b and STAT1. Simultaneous knockdown of STAT5b and STAT1 had no additional inhibitory effect on migration (Figure 19A). Although only performed once, this gave an initial indication that STAT5b and STAT1 may function in the same pathway. In BT-549 cells, we detected some STAT1 co-immunoprecipitated with endogenous STAT5b (Figure 19B). This association was also seen in GST-STAT5b pulldown assays (Figure 19C). It is of note that there was some non-specific association of STAT1 with GST alone, albeit much less than that associated with GST-STAT5b. Attempts to eliminate this non-specific association were not successful. Furthermore, endogenous association was not consistently observed.

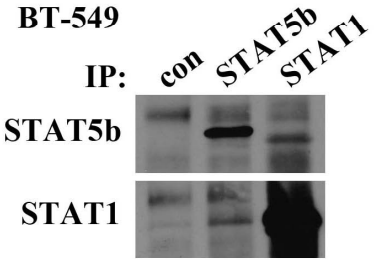
Figure 19: Examining STAT5b-STAT1 Heterodimer Formation

(A) MDA-MB-231 cells were left untransfected (con), or transfected with siRNA targeted to luciferase (siLuc), STAT5b, STAT1, or both STAT5b and STAT1. Seventy-two hours following transfection, transwell migration assays were performed with 10% FBS in the lower chambers for 6 hours as previously described. Results are graphed as average number of migratory cells per field $\pm$ SE (n=1). (B) BT-549 cells were lysed from growing conditions and immunoprecipitated with antibodies specific for STAT5b, STAT1, or pre-immune serum from STAT5b antibody production (con). Immunoprecipitated proteins were blotted with STAT5b (*top*) and STAT1 (*bottom*) antibodies. (C) Lysates from BT-549 cells in normal growth conditions were incubated with GST- or GST-STAT5b conjugated glutathione agarose beads and immunoblotted with antibodies specific for STAT5b (*top, middle*) or STAT1 (*bottom*). Whole cell lysates were analyzed as input controls (*right*).

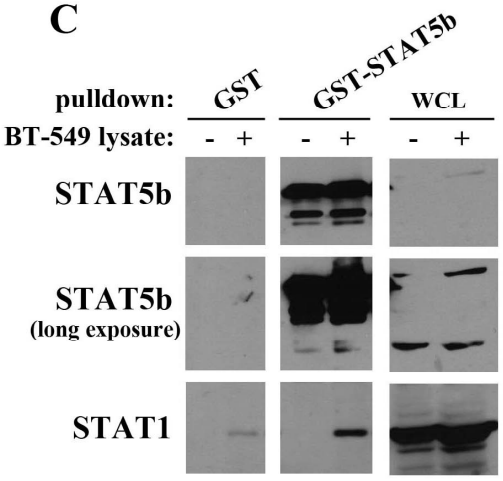
A


MDA-MB-231
average cells/field
250
200
150
100
50
0
con
siLuc
siSTAT5b
siSTAT1
siSTAT5b/1

B


BT-549
IP:
con
STAT5b
STAT1
STAT5b
STAT1

C


pulldown:
GST
GST-STAT5b
WCL
BT-549 lysate:
- + - + - +
STAT5b
STAT5b
(long exposure)
STAT1

C. Determine whether knockdown of STAT5b affects speed and direction of wound closure

STAT5b Knockdown Results in Multiple Protrusions and Negatively Affects Directionality During Wound Closure

Wound healing assays were performed to examine cell morphology and motility during wound closure of cells plated on FN. Following STAT5b knockdown, wounds were allowed to close for 6 hours and time lapse microscopy was used to track cell movement into the wound. Figure 20A depicts the path of cells (tracked by single cell nuclei) along the wound edge. Control cells migrated in relatively straight lines into the wound area. In contrast, STAT5b knockdown cells showed compromised directionality in movement, migrating both horizontally and vertically. To quantitate these results, we measured migration rate and directional persistence. Knockdown of STAT5b had little effect on overall migration rate. However, cells deficient for STAT5b exhibited significant loss of directional persistence (30% decrease compared to control cells), suggestive of a defect in cell polarity (Figure 20).

A hallmark of cell polarization during migration is re-localization of the Golgi apparatus between the nucleus and leading edge, which allows transport of newly synthesized proteins to the leading edge (Kalt and Schliwa, 1993; Nobes and Hall, 1999). STAT5b knockdown cells had a defect in polarity, illustrated by a decrease in the number of cells with properly re-oriented Golgi (Figure 21B). In many knockdown cells, the Golgi localized between the nucleus and tail. In cells with multiple protrusions, the Golgi could often be found divided between the nucleus and each protrusive edge. Representative images are shown in Figure 21A and quantitation of cell number is shown in Figure 21B.

Figure 20: STAT5b Knockdown Negatively Affects Directionality During Wound Closure

Wounds were made in confluent monolayers of siRNA-transfected BT-549 cells plated onto FN-coated dishes (3µg/ml), and time lapse microscopy was used to track cell movement into the wound over 6 hours. (A) Progressive line images tracking single cell nuclei of cells along the wound edge. Two independent experiments are shown. (B) Migration rate (µm/h) was calculated by dividing the length of the migration path by the total movie time. (C) Directional persistence (D/T) was determined as the net displacement (D) divided by the total length of the migration path (T). (B, C) Values from at least four independent experiments were used for calculations (siLuc, n=24; siSTAT5b, n=32). Student's *t* test was used to determine statistical significance between siLuc and siSTAT5b. Directional persistence: *, $p<0.01$. Comparisons of migration rate were not statistically significant.

** Figure 4 of manuscript*

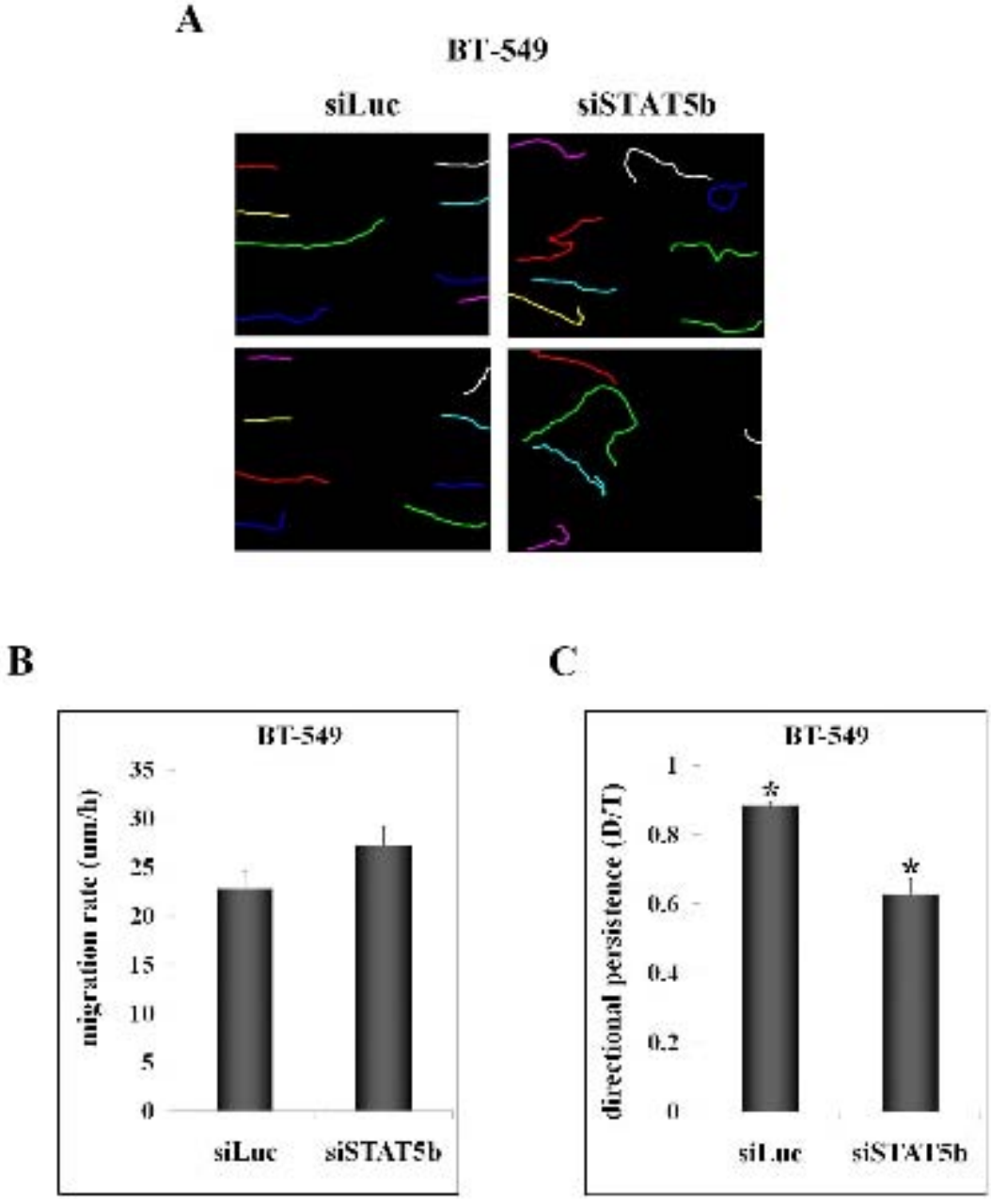
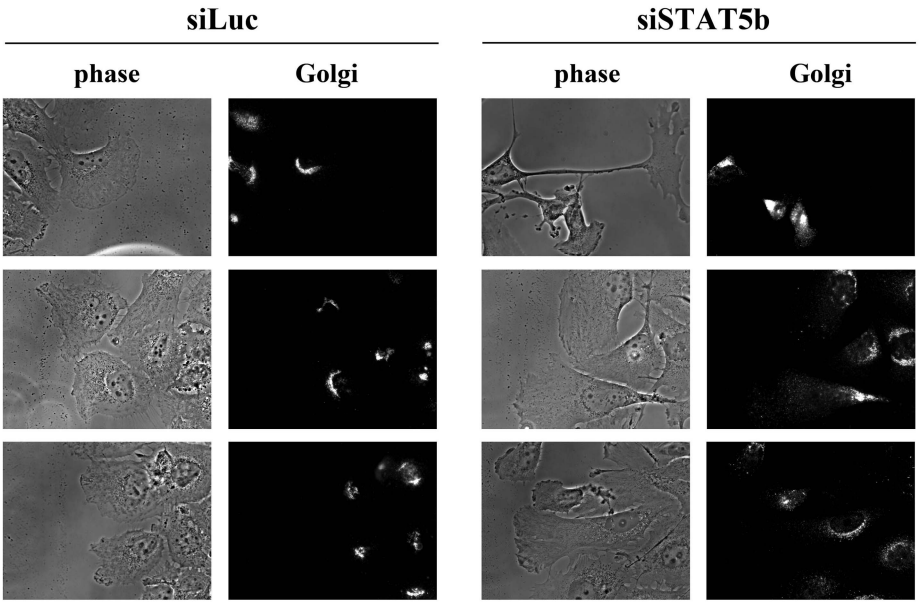

A
BT-549
siLuc
siSTAT5b
B
BT-549
migration rate (um/h)
35
30
25
20
15
10
5
0
siLuc
siSTAT5b
C
BT-549
directional persistence (D/T)
1
0.8
0.6
0.4
0.2
0
*
*
siLuc
siSTAT5b

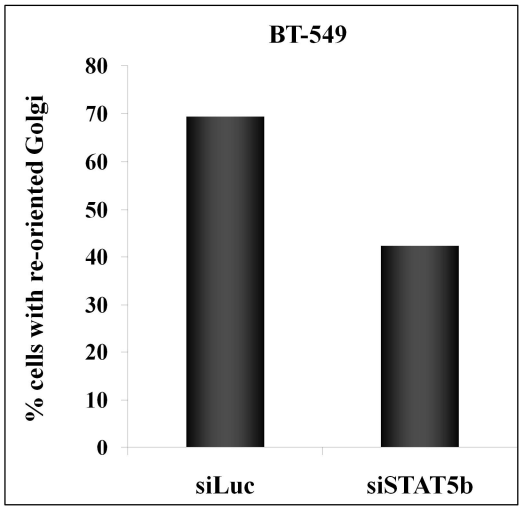
Figure 21: STAT5b Knockdown Affects Golgi Localization During Wound Healing

(A) BT-549 cells were transfected with siLuc or siSTAT5b siRNA. Seventy-two hours post-transfection, confluent monolayers of cells plated on FN (3µg/ml) were wounded. Wounds were allowed to heal for 6 hours, after which time cells were fixed, permeabilized, and stained for Golgi bodies using an antibody specific to the Golgi matrix protein GM130. Phase (*left*) and fluorescence (*Golgi, right*) pictures of cells along the wound edge were taken at 20x magnification. (B) Graph represents percentage of cells with Golgi re-oriented between the nucleus and leading edge. Approximately 70-85 cells were counted for each condition (n=1).

A


BT-549
siLuc
siSTAT5b
phase
Golgi
phase
Golgi

B


BT-549
% cells with re-oriented Golgi
80
70
60
50
40
30
20
10
0
siLuc
siSTAT5b

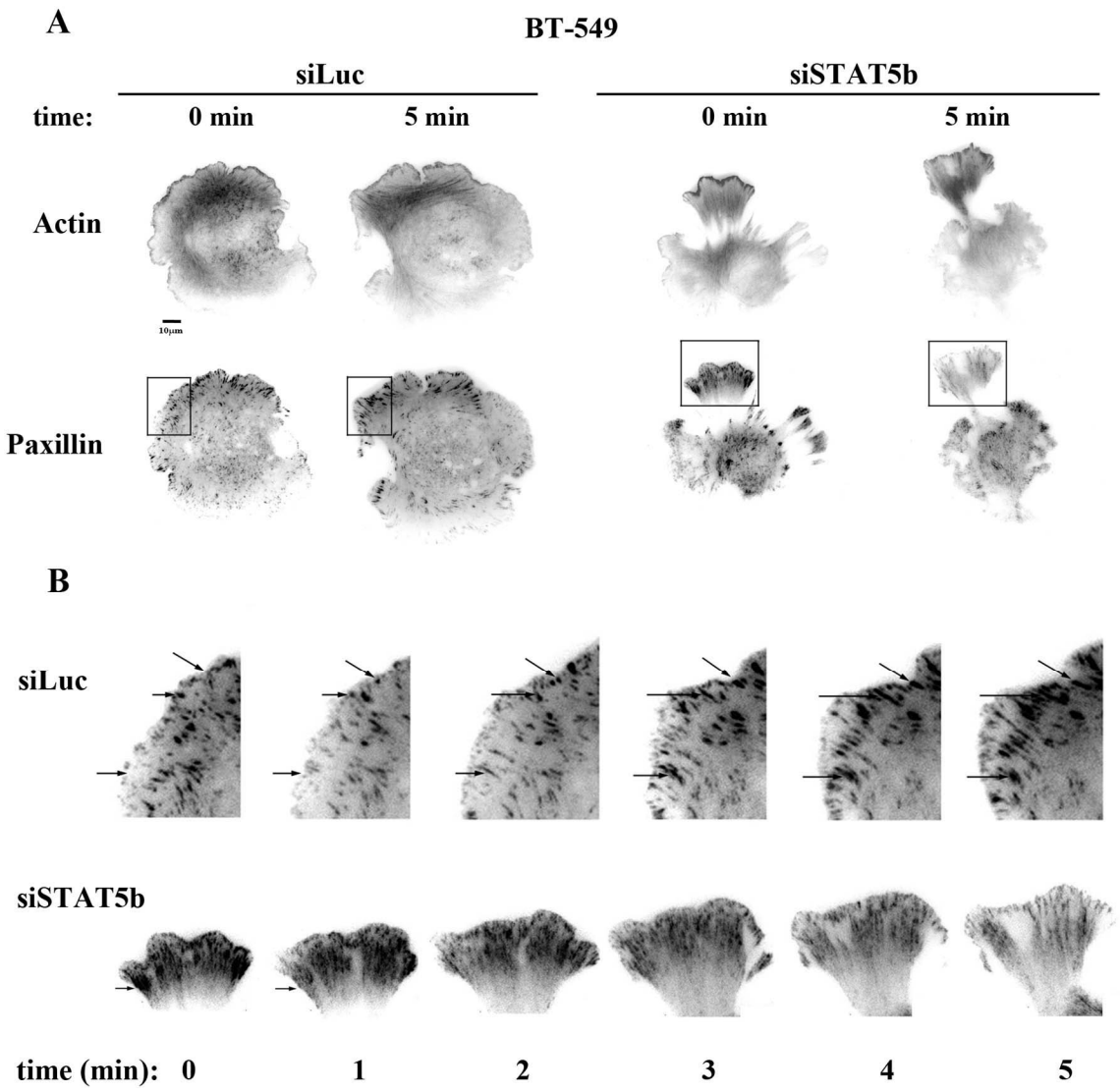
D. Establish whether knockdown of STAT5b affects focal adhesion formation and/or turnover

To examine focal adhesion and actin dynamics at the membrane, total internal reflection fluorescence (TIRF) microscopy was employed to image cells plated on FN (Figure 22). Spreading rates were calculated as the total distance spread (μm) divided by the total time of filming (min). STAT5b knockdown cells spread slightly faster on FN and exhibited a striking phenotype characterized by the formation of multiple irregular protrusions (Figure 22A). Knockdown cells were highly contractile and displayed increased retrograde movement of cell adhesions. Analysis of focal adhesion turnover revealed that control cells undergo normal focal adhesion turnover with small adhesions present at the leading edge and mature, stable adhesions located further inside the cell (Figure 22B). STAT5b knockdown cells readily form focal adhesions at the leading edge of protrusive lamellipodia, but over time these lamellipodia contract and the adhesions undergo retrograde flow back into the cell (Figure 22B). These data indicate that STAT5b may contribute to the coordinated regulation of protrusive and contractive processes during cell migration.

Figure 22: STAT5b Knockdown Cells Exhibit Multiple Protrusions and Increased Focal Adhesion Retraction

BT-549 cells were transfected with siRNA (siLuc or siSTAT5b), GFP-actin, and mKO-paxillin. Seventy-two hours post-transfection, cells were plated on 3μg/ml FN for 20-30 minutes and TIRF time lapse microscopy was performed. Images were taken every 3 seconds for 5 minutes at 60x magnification. *Line indicates 10 μm*. (A) Still images representing actin and paxillin staining in control (siLuc) and STAT5b knockdown (siSTAT5b) cells at start of filming (0 min) and end of filming (5 min). (B) Enlarged images depicting boxed areas from part A. Arrows identify single focal adhesions at each time point. (C) Protrusion rate (μm/min) was calculated as the length of the protrusion divided by the total time of the movie for at least 3 independent experiments (siLuc, n=16; siSTAT5b, n=13). Student's *t* test was used to determine statistical significance between siLuc and siSTAT5b *, $p<0.01$.

** Figure 5 of manuscript*


A
BT-549
siLuc
siSTAT5b
time:
0 min
5 min
0 min
5 min
Actin
Paxillin
10μm
B
siLuc
siSTAT5b
time (min): 0 1 2 3 4 5
C
BT-549
protrusion rate (um/min)
2
1.5
1
0.5
0
*
*
siLuc
siSTAT5b

E. Examine STAT5b activation and signaling upon adhesion

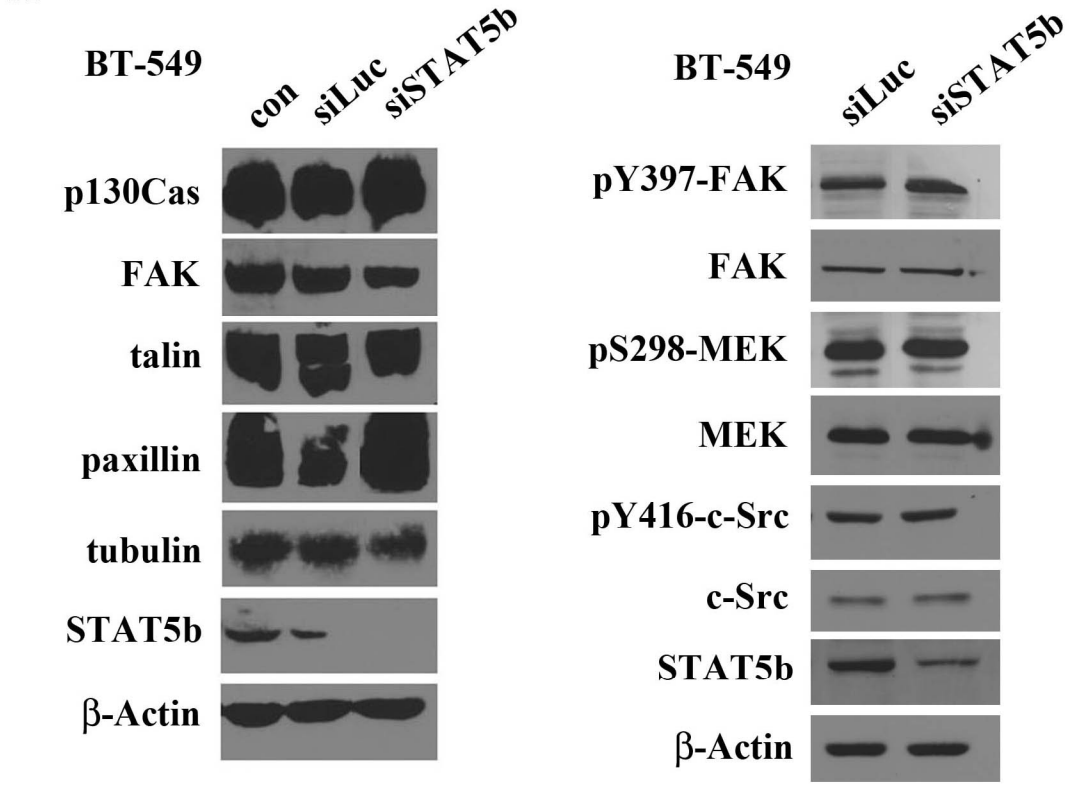
Expression and Activation of Migratory Signaling Proteins in STAT5b Knockdown Cells

The signaling pathways downstream of integrin engagement which control lamellipodial protrusion and contraction are well established (Figure 4). In order to pinpoint where STAT5b may be acting, we examined expression levels and activation status of proteins in these pathways (Figure 23). We observed no change in expression or activation of p130Cas, focal adhesion kinase (FAK), mitogen-activated protein kinase kinase (MEK), c-Src, talin, paxillin, or tubulin following STAT5b knockdown. However, a preliminary experiment did show changes in myosin light chain (MLC) phosphorylation. In this experiment, STAT5b knockdown cells maintained high levels of myosin light chain phosphorylation when placed in suspension, suggestive of dysregulated Rho signaling (Figure 23B). This experiment must be repeated to confirm the observed effect.

Although STAT5b knockdown did not affect overall expression of p130Cas, we did observe association between STAT5b and p130Cas upon attachment of MDA-MB-231 cells to both FN and plastic control (Figure 24A). Additionally, endogenous p130Cas from both BT-549 and MDA-MB-231 cells associated with GST-tagged recombinant STAT5b in GST pulldown assays (Figure 24B,C). We did not observe association between STAT5b and FAK, another focal adhesion protein, nor non-specific association of p130Cas with GST alone. Furthermore, this association was not dependent on attachment to fibronectin as p130Cas isolated from cells attached to plastic also associated with GST-STAT5b. Further experiments must be performed to confirm association between STAT5b and p130Cas.

Figure 23: Examining Expression and Activation of Migratory Signaling Proteins in STAT5b Knockdown Cells

(A) Seventy-two hours following siRNA transfection, BT-549 cells were lysed (*left*) or plated in serum-free media onto FN-coated plates (2μg/ml) for 1 hour and then lysed (*right*). Whole cell lysates were immunoblotted with antibodies specific for p130Cas, FAK, pY397-FAK, talin, paxillin, tubulin, MEK, pS298-MEK, c-Src, pY416-c-Src, STAT5b, and β–actin as a loading control. Western blots from two independent experiments are shown. (B) Seventy-two hours following siRNA transfection, BT-549 cells were plated in serum-free media onto FN-coated plates (2μg/ml) or put in suspension for 1 hour. Whole cell lysates were immunoblotted with antibodies specific for MLC, pS19-MLC, STAT5b, and β–actin as a loading control.


A
BT-549
con
siLuc
siSTAT5b
p130Cas
FAK
talin
paxillin
tubulin
STAT5b
β-Actin
BT-549
siLuc
siSTAT5b
pY397-FAK
FAK
pS298-MEK
MEK
pY416-c-Src
c-Src
STAT5b
β-Actin

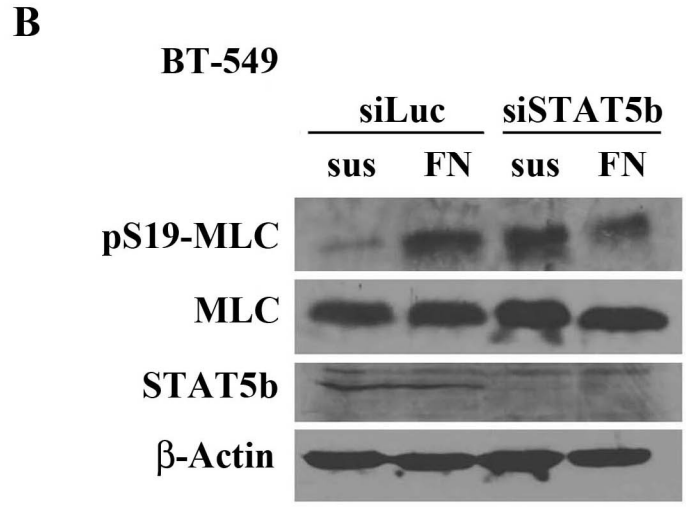
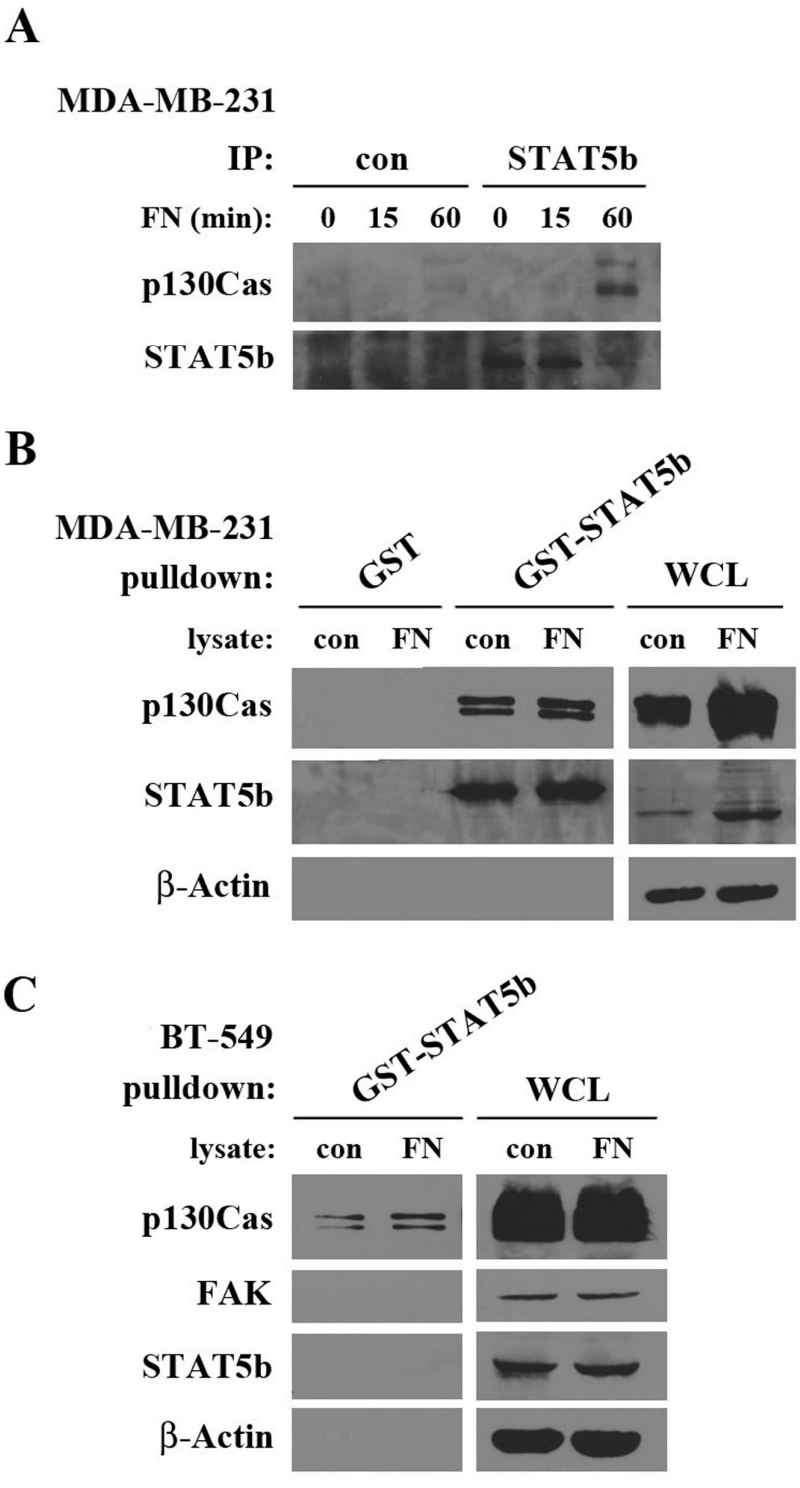

B
BT-549
siLuc
siSTAT5b
sus
FN
sus
FN
pS19-MLC
MLC
STAT5b
β-Actin

Figure 24: Examining STAT5b Association with p130Cas

(A) MDA-MB-231 cells were lysed from growing conditions (FN-0 min) or plated onto FN-coated plates (2μg/ml) in serum-free media for 15 min or 1 hour prior to lysis. Lysates were immunoprecipitated with a STAT5b-specific antibody or pre-immune serum from STAT5b antibody production (con). Immunoprecipitated proteins were blotted with p130Cas and STAT5b antibodies. (B, C) MDA-MB-231 or BT-549 cells were plated onto uncoated (con) or FN-coated (2μg/ml) dishes for 1 hour in serum-free media and lysed. Lysates were incubated with GST- or GST-STAT5b conjugated glutathione agarose beads and immunoblotted with antibodies specific for p130Cas, FAK, STAT5b, and β-Actin. Whole cell lysates were run as input controls (*right*).


A
MDA-MB-231
IP:
con
STAT5b
FN (min):
0 15 60 0 15 60
p130Cas
STAT5b
B
MDA-MB-231
pulldown:
GST
GST-STAT5b
WCL
lysate:
con FN con FN con FN
p130Cas
STAT5b
β-Actin
C
BT-549
pulldown:
GST-STAT5b
WCL
lysate:
con FN con FN
p130Cas
FAK
STAT5b
β-Actin

Discussion

STAT5b is traditionally thought of as a pro-proliferative, pro-survival transcription factor, and minimal research has been done examining its role in cell migration. We originally set out to investigate the role of EGF-induced STAT5b signaling in EGFR-overexpressing breast cancer cells. Although BT-549 cells migrated chemotactically toward serum (Figure 7), no migration was seen to EGF alone in these cells, or in EGFR-overexpressing MDA-MB-231 breast cancer cells (*data not shown*). In the presence of an equal serum gradient, we did observe an increase in migration to EGF at concentrations of 0.1-10ng/ml EGF (Figure 8). Interestingly, increasing the concentration of EGF to 100ng/ml decreased migration to basal levels. Inhibitory effects of higher EGF concentrations on EGFR-overexpressing breast cancer cells have been reported in the past. Specifically, treatment of MDA-MB-468 breast cancer cells with 10 or 100ng/ml EGF suppressed proliferation and induced apoptosis (Armstrong et al., 1994). Moreover, EGF promoted migration of MDA-MB-231 cells at concentrations less than 50ng/ml, but had no effect on migration at higher concentrations (Price et al., 1999). This is consistent with our observations in BT-549 cells. Since we could not recapitulate significant EGF-induced migration of either cell line in the absence of serum, we ruled out EGF as the primary chemotactic stimulus responsible for serum-induced migration of these cells, and used total serum as the chemotactic stimulus for future knockdown studies.

Knockdown of STAT5b inhibited migration of both BT-549 and MDA-MB-231 breast cancer cell lines to serum (Figure 9). Moreover, knockdown of STAT5b inhibited breast cancer cell migration independent of serum concentration (Figure 11). Nonetheless, migration was not completely abrogated upon knockdown of STAT5b in either cell line. The cells retained their capacity to migrate at a similar level across all serum concentrations tested. This result suggests

that there may be another pathway, independent of STAT5b signaling, that mediates migration of breast cancer cells to serum. Knockdown of STAT5b also inhibited invasion of BT-549 cells (Figure 10). Invasion assays in MDA-MB-231 cells were not successful. This may be due to sub-optimal experimental conditions. However, given the robust migration seen in both cell lines, we chose to concentrate on STAT5b's role in cell migration rather than invasion.

Transient overexpression of STAT5b did not alter migratory counts in BT-549 cells (Figure 12). However, we cannot conclude that overexpression of STAT5b has no effect on migration, as this observed lack of effect may be due to low transfection efficiency. Although immunoblots confirm overexpression of STAT5b (Figure 12B), we are not able to differentiate as to whether this is a result of significant STAT5b overexpression in a large number of cells, or high overexpression of STAT5b in a small number of cells. To circumvent the latter possibility, transfected cells only should be counted in future experiments.

Transwell migration assays were performed for 3-6 hours and therefore, loss of proliferation during the assay can be ruled out as an explanation for the decrease in migratory cell number. While STAT5b knockdown did influence cell cycle distribution, these effects were minimal, differed between the two cell lines tested, and did not correlate with the decrease in migration observed (Figure 13). There was no significant change in viability with STAT5b knockdown after 6 hours of plating, and thus, cell death during the assay can also be ruled out (Table 2). Lastly, the number of adherent cells after 6 hours was similar in control and knockdown cells (Table 2). However, a time course of adherence was not performed. Therefore, while it can be concluded that there is no overall defect in adherence after 6 hours, the possibility that adherence is delayed or less efficient initially cannot be ruled out. It is of note that during the seventy-two hours leading up to the assay, there was a decrease in cell number in

STAT5b knockdown cells compared to control, indicating that there are long-term proliferation and/or survival effects from loss of STAT5b. This result is not surprising given that STAT5b regulates transcription of pro-proliferative and pro-survival genes.

In addition to STAT5b knockdown, STAT1 knockdown also inhibited migration of MDA-MB-231 cells to serum. Despite incomplete knockdown of STAT1 protein, migration was inhibited to the same level as that seen with near complete knockdown of STAT5b protein (Figure 14). Given the evidence for STAT1 as a tumor suppressor, these results were unexpected. STAT1 signaling induces cell cycle arrest and apoptosis *in vitro* (Haura et al., 2005). Additionally, mice lacking STAT1 have a higher incidence of tumor formation following exposure to carcinogens, and a higher rate of spontaneous tumor formation in the absence of p53 (Kaplan et al., 1998). However, analysis of STAT1 in primary tumor samples is not as clear-cut as *in vitro* and *in vivo* data. Up-regulation of STAT1 levels, tyrosine phosphorylation, and serine phosphorylation have been reported in leukemia and cervical cancer (Frank et al., 1997; Hudelist et al., 2005; Spiekermann et al., 2001). Therefore, the role of STAT1 in tumorigenesis may be cell-type and context dependent.

One explanation for the observation that knockdown of either STAT1 or STAT5b has similar effects on migration of MDA-MB-231 cells is that STAT1 and STAT5b are signaling through the same pathway. Given that endogenous STAT1 could not compensate for loss of STAT5b and vice-versa (Figures 14, 19), we explored the possibility that STAT1-STAT5b heterodimers are responsible for promoting migration of these cells. We were able to detect both endogenous and exogenous association of STAT1 and STAT5b but the association was weak and inconsistent (Figure 19). In theory, STAT proteins should have the ability to heterodimerize. However, there are limited studies on heterodimerization, with most reporting STAT1-STAT3

and STAT5a-STAT5b heterodimers (Cella et al., 1998; Han et al., 1997; Rosenthal et al., 1997; Schulze et al., 2000). There has only been one report of STAT1-STAT5b heterodimerization, in which DNA-bound heterodimers were detected in leukemia cells (Brizzi et al., 1999). The lack of significant STAT1-STAT5b association is consistent with our data illustrating that STAT5b Y699 phosphorylation and subsequent active dimer formation and transcriptional activity are not required for migration (Figure 18).

Contrary to STAT1 and STAT5b, STAT3 and STAT5a were not necessary for migration of MDA-MB-231 breast cancer cells (Figure 14). Despite strong evidence of a requirement for STAT3 in prostate cancer cell migration, there is limited data on the role of STAT3 in breast cancer migration (Azare et al., 2007; Zhou et al., 2006). Treatment of MCF-7 breast cancer cells with the cytokine oncostatin M (OSM) induced migration, and this migration was inhibited by dominant-negative STAT3 (Zhang et al., 2003). However, dominant-negative STAT3 had no effect on basal migration of these cells or chemotaxis toward OSM. These data are in agreement with a study showing that neither wild-type nor dominant-negative STAT3 affected migration of T-47D breast cancer cells to interleukin-6 (IL-6) or OM (Badache and Hynes, 2001). These studies suggest that while STAT3 mediates intrinsic cell migration following cytokine stimulation, it is not necessary for chemotaxis toward a cytokine stimulus. Although these studies were both performed in ER-positive breast cancer cell lines, they support our findings in ER-negative, EGFR-overexpressing breast cancer cells, that STAT3 is not required for chemotactic migration to serum.

Our results showing that knockdown of STAT5a had no effect on migration of MDA-MB-231 cells seem contradictory to those published by *Sultan, et al*. (Sultan et al., 2005). However, in those studies, STAT5a was overexpressed in the moderately migratory BT-20 and

T47D breast cancer cell lines which contain little to no endogenous STAT5a. The STAT5a and STAT5b expression pattern in those cell lines is very different than that seen in MDA-MB-231 cells (which express substantial levels of both STAT5a and STAT5b), and consequently, the signaling may differ. Moreover, the role of endogenous STAT5b was not investigated in *Sultan, et al.'s* studies. We and others have found that STAT5b is the predominant STAT5a/b protein expressed in breast cancer cell lines and tissues, and that STAT5b, not STAT5a, mediates proliferation of breast cancer cells (Kloth et al., 2003; Sultan et al., 2005; Weaver and Silva, 2006). This dichotomy between STAT5a and STAT5b has also been observed in squamous cell cancer of the head and neck (SCCHN) and prostate cancer. In tumor tissues from patients with SCCHN, STAT5b is overexpressed and phosphorylated, but STAT5a levels and phosphorylation are similar to that seen in control tissues (Xi et al., 2003b). In addition, antisense inhibition of STAT5b, but not STAT5a inhibited growth of SCCHN xenografts (Xi et al., 2003b). While both STAT5a and STAT5b are activated by Src, only STAT5b accelerated v-Src-mediated transformation of fibroblasts, and increased v-Src-induced growth, survival, and motility of these cells (Kazansky and Rosen, 2001). Furthermore, STAT5b, but not STAT5a, is overexpressed in the metastatic C2H prostate cancer cell line, and introduction of dominant-negative STAT5b into these cells inhibited cell cycle progression, invasion, and *in vivo* tumor growth (Kazansky et al., 2003). As there is precedence for differing functions of STAT5a and STAT5b in both cancerous and noncancerous cells, it is imperative to determine the individual contributions of these proteins to a given process.

The data presented here are consistent with previous cell line and animal studies which have established a role for STAT5b in promoting tumor growth and progression *in vitro*. However, studies of primary breast tumors are not as straightforward. *Cotarla, et al.* reported

constitutive activation of STAT5a/b in approximately 76% (n=83) of human breast tumors analyzed (Cotarla et al., 2004). However, in these tumors, activated STAT5a was associated with differentiation. This positive correlation between STAT5a/b phosphorylation and tumor differentiation was confirmed by a second, larger study of more than 500 tumor samples (Yamashita et al., 2006). Likewise, in lymph node negative breast cancer, STAT5a/b expression is associated with a better prognosis (Nevalainen et al., 2004). Nevertheless, aggressive, more advanced breast tumors, such as triple-negative breast tumors have not been analyzed. Our studies in ER-negative, EGFR-overexpressing breast cancer cells demonstrate an integral pro-proliferative role for STAT5b downstream of the EGFR (Kloth et al., 2002; Kloth et al., 2003; Weaver and Silva, 2006). EGF stimulation of ER negative, EGFR-overexpressing SKBr3 breast cancer cells induced STAT5b-mediated transcription and subsequent DNA synthesis (Kloth et al., 2003; Weaver and Silva, 2006). Interestingly, addition of STAT5a to these cells abrogated EGF-induced DNA synthesis (Weaver and Silva, 2006) suggesting that STAT5a and STAT5b may have distinct, opposing roles in EGFR-overexpressing breast tumors, including triple-negative tumors.

Having established the necessity of STAT5b for maximal migration of breast cancer cells to serum, we next investigated the mechanism by which this occurs. We utilized knockdown-rescue experiments to determine which regions/functions of STAT5b are necessary for maximal migration to serum. Equivalent rescue of migration was achieved with co-transfection of either wt-, Y699F- or dn-STAT5b (Figure 18). Phosphorylation of Y699 is a hallmark of STAT5b transcriptional activity. If this residue is mutated to a phenylalanine such that it cannot be phosphorylated (Y699F), the resulting STAT5b mutant is transcriptionally inactive (Fox et al., 2008; Weaver and Silva, 2007b). Dn-STAT5b is also transcriptionally inactive due to loss of the

TAD and the ability to bind transcriptional cofactors (Kloth et al., 2003). The ability of Y699F- and dn-STAT5b to rescue migration implies that STAT5b transcriptional activity is not required for migration. Moreover, wt-STAT5b retained its ability to rescue migration in the presence of the transcriptional inhibitor actinomycin D, further supporting this conclusion (Figure 17).

Interestingly, the S731A-STAT5b mutant was not able to rescue migration (Figure 18). In addition to tyrosines, STAT proteins contain C-terminal serine residues which can be phosphorylated in response to cytokines and growth factors (Decker and Kovarik, 2000; Park et al., 2001; Weaver and Silva, 2007a). While serine phosphorylation is necessary for maximal transcriptional activity of STAT1 and STAT3, the consequence of serine phosphorylation on STAT5b activity is poorly understood (Decker and Kovarik, 2000). Furthermore, the impact of S731 phosphorylation independent of transcription has not been examined. Therefore, the significance of this phosphorylation event is not known at this time.

Similar to S731A-STAT5b, R618K-STAT5b also did not rescue migration following STAT5b knockdown. This STAT5b mutant contains a point mutation in the conserved SH2 domain arginine required for binding phosphorylated tyrosines (Chen et al., 1998; Heim et al., 1995). Although STAT proteins are predominantly thought of as transcription factors which function in the nucleus, recent work has identified a non-transcriptional, cytoplasmic role for unphosphorylated STAT3 in regulating tubulin dynamics (Ng et al., 2006). Unphosphorylated STAT dimers take on an antiparallel configuration through interactions between DNA-binding domains (Mao et al., 2005; Neculai et al., 2005). In this conformation, the SH2 domains are on opposite sides of the structure and are free to interact with phosphorylated tyrosines of other proteins. The necessity of STAT5b's SH2 domain in migration is consistent with a cytoplasmic function of unphosphorylated STAT5b. From these data, we propose that following integrin

engagement, STAT5b utilizes its SH2 domain to act as an adaptor protein, bringing together signaling molecules necessary for efficient, directional migration. There are many tyrosine phosphorylated proteins in the cytoplasm involved in migratory signaling with which STAT5b may interact, and future studies are aimed at uncovering these associations.

The work presented here establishes an integral role for STAT5b in breast cancer cell migration. Migration is a complex event that requires multiple, interdependent processes: polarization; cell-matrix attachment and adhesion turnover; cytoskeletal changes; and actin-myosin contraction. While transwell migration assays are useful for evaluating overall migration of a population of cells, they do not allow for analysis of the individual processes essential for proper migration. To examine cell movement following STAT5b knockdown in more detail, we performed live imaging of individual cells in real-time. During wound healing, the Golgi apparatus did not effectively re-orient in STAT5b knockdown cells and directional persistence was lost (Figures 20, 21). Additionally, during spreading, STAT5b knockdown cells formed multiple protrusions exhibiting increased contractility (Figure 22). Taken together, these data support a role for STAT5b in multiple migratory processes: polarity, protrusion, and contraction.

Small GTPases and adhesion-dependent kinases are critical regulators of the migratory process. Our preliminary studies examining FAK, Src, p130Cas, paxillin, and talin in STAT5b knockdown cells showed no changes in expression levels and/or phosphorylation (Figure 23), suggesting that STAT5b is acting downstream of these signaling molecules. Attempts to measure small GTPase activity were unsuccessful. However, the STAT5b knockdown phenotype characterized by loss of polarity, increased membrane protrusions, and increased contractility, is consistent with dysregulated GTPase signaling. While the mechanisms responsible for maintaining cell polarity are not completely understood, antagonism between

Rho and Rac signaling is believed to play an important role (Vicente-Manzanares et al., 2005). Additionally, both actin filament assembly and acto-myosin contraction are regulated by the small GTPases Rho, Rac, and Cdc42. Rho induces actomyosin contraction through activation of Rho-kinase (ROCK), and subsequent ROCK-mediated phosphorylation of myosin light chain (MLC) (Hirata et al., 2009; Kimura et al., 1996). Rac also regulates contraction through p21-associated kinase (PAK). PAK phosphorylates myosin light chain kinase (MLCK), inhibiting MLCK, resulting in decreased MLC phosphorylation and decreased contractility (Sanders et al., 1999). Moreover, Rho, Rac, and Cdc42 stimulate actin nucleating proteins, facilitating actin filament elongation and subsequent membrane protrusion (Pollard and Borisy, 2003; Vartiainen and Machesky, 2004; Watanabe and Higashida, 2004). As a whole, our data showing that loss of STAT5b affects cell polarity, membrane protrusion, and contractility, supports a potential function for STAT5b in the regulation of GTPase signaling, thereby impacting multiple migratory processes.

In summary, these studies are the first to report a role for STAT5b in migration of breast cancer cells. It is well established that STAT5b positively regulates breast cancer cell proliferation and survival, two processes important for initial tumor formation and growth. Our data implicates STAT5b in the later stages of tumorigenesis also, specifically cell migration. Future studies will further elucidate the mechanism by which STAT5b exerts its effect on migration, thereby broadening our understanding of how STAT5b promotes tumorigenesis and possibly metastasis. These studies would facilitate the long-term goal of defining conditions whereby STAT5b would be an effective therapeutic target for the treatment of breast cancer.

Materials and Methods

Cell culture – BT-20, BT-549, MDA-MB-231, MDA-MB-468, and SKBr3 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). 21PT and 21MT-1 human breast cancer cell lines were a kind gift from Dr. Vimla Band (University of Nebraska, Omaha, Nebraska). Cells were passaged twice per week and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All tissue culture reagents were purchased from Invitrogen (Gaithersburg, MD).

siRNA Transfection - BT-549 and MDA-MB-231 cells were transfected with siGENOME SMARTpool siRNA targeting human STAT5b, STAT1, or STAT3, individual custom oligonucleotides specific for STAT5a or STAT5b (siGENOME STAT5b SMARTpool duplex #3), or luciferase duplex control, all purchased from Dharmacon (Lafayette, CO). Transfections were performed using Oligofectamine (Invitrogen) or Amaxa nucleofection (Amaxa/Lonza, Walkersville, MD) using solution T and program X-013 (MDA-MB-231) or A-023 (BT-549) as per manufacturers' instructions. For transient overexpression studies, BT-549 cells were transfected with hemagglutinin (HA) vector, HA-STAT5a, or HA-STAT5b using Lipofectamine Plus (Invitrogen) according to manufacturer's instructions. For knockdown-rescue experiments, cells were transfected simultaneously with siSTAT5b SMARTpool duplex #3 and hemagglutinin (HA)-tagged wild-type-, Y699F-, dn-, R618K-, S731A-, or myc-tagged 1-144- STAT5b engineered to be immune to knockdown by introduction of four silent point mutations in the siRNA target sequence. These point mutations were introduced using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) and constructs were sequenced to verify mutations.

Transwell Migration and Invasion Assays - BT-549 and MDA-MB-231 cells were transfected with siRNA as described above. Seventy-two hours post-transfection, different numbers of BT-549 cells (as indicated) or $1x10^5$ MDA-MB-231 cells were plated in serum-free media (DMEM + 0.1% BSA (bovine serum albumin)) into the upper chambers of BD BioCoat Control Chamber transwell plates (BD Biosciences, San Jose, CA). Various concentrations of FBS were placed in the lower chambers. For EGF studies, 1% FBS was placed in the upper and lower chambers and EGF (0.1-100ng/ml) was placed in the lower chambers only. Plates were incubated at 37°C and migration was allowed to proceed for 3-6 hours. For invasion assays, $2.5x10^4$ cells were plated in BD BioCoat Matrigel Invasion Chamber transwell plates (BD Biosciences) forty-eight hours post-transfection, and incubated at 37°C for 24 hours. After incubation, non-migratory or non-invasive cells in the upper chambers were removed with cotton swabs, and the remaining cells were stained with 0.1% crystal violet (Sigma, St. Louis, MO) in 20% ethanol. Cells were counted using a Zeiss Invertoskop light microscope and graphed as average cells per field, average cells per filter, or relative migration/invasion compared to control. For actinomycin D experiments, cells were pre-treated for 1 hour with DMSO (control) or 10μg/ml actinomycin D (Thermo Fisher Scientific, Pittsburgh, PA) and treatment was left on for the duration of the assay. For STAT5b SH2 inhibitor studies, cells were pre-treated for 1 hour with indicated concentrations of STAT5 inhibitor (Calbiochem, San Diego, CA), and treatment was continued for the duration of the assay. For β1 integrin blocking experiments, cells were pre-treated for 1 hour with DMSO (control), mouse IgG antibody control (control) or 10μg/ml monoclonal anti-human integrin β1 antibody (R&D Systems, Minneapolis, MN), and treatment was continued for the duration of the assay. For migration to chemotactic stimuli, 100ng/ml SDF-1α or SDF-1β (Peprotech, Rocky Hill, NJ), 100ng/ml CCL21 (Peprotech), or 2μM LPA (BIOMOL

International, Plymouth Meeting, PA) was placed in lower chambers in serum-free media. Alternatively, newborn calf serum was charcoal-stripped and placed in lower chambers. For migration to extracellular matrix components, the undersides of filters were coated with 0-10μg/ml human plasma fibronectin (FN) (BD Biosciences) or recombinant human vitronectin (VN) (R&D Systems) overnight at 4°C, as indicated, and DMEM/0.1%BSA was placed in both the upper and lower chambers.

Trypan Blue Assays - Seventy-two hours following siRNA transfection, $4x10^5$ cells were transferred to new plates containing serum-free media for 6 hours at 37°C. Media was collected, cells were washed in phosphate-buffered saline (PBS), and the media and washes were combined to isolate non-adherent cells. Adherent cells were trypsinized and collected separately. Both fractions were stained with trypan blue (Fisher, Herndon, VA) and the total number of cells and number of trypan blue positive cells were counted via hemacytometer.

Propidium Iodide Staining and Flow Cytometry - Approximately $1x10^6$ cells were collected and fixed in 70% ethanol for two hours on ice. Fixed cells were washed in PBS and stained in a solution of 20μg/ml (MDA-MB-231 cells) or 40μg/ml (BT-549 cells) propidium iodide (Sigma) in 0.1% Triton X-100 containing 200μg/ml DNase-free RNase A (Qiagen, Germantown, MD) for 1 hour at room temperature. Cells were analyzed on a FACSCalibur bench cytometer (Becton Dickinson, San Jose, CA) using ModFit software.

Glutathione S-Transferase (GST) Pull-downs – GST and GST-STAT5b were made in our lab as previously described (Weaver and Silva, 2007b). BT-549 cells were lysed in GST lysis buffer

[20mM NaCl, 20mM Tris, pH 8.0, 1mM EDTA, 0.5% Igepal]. Lysates were pre-cleared by incubation with GST for 1 hour at 4°C followed by incubation with glutathione agarose beads (Amersham/GE Healthcare, Piscataway, NJ) for 1 hour at 4°C. GST or GST-STAT5b was conjugated to glutathione agarose beads for 2 hours at 4°C and then lysates were added for 2 hours at 4°C. Glutathione agarose pellets were washed three times in lysis buffer and bound proteins were removed by boiling in 2x Laemmli buffer containing β-mercaptoethanol or 20mM dithiotreitol (DTT) for 5 minutes at 100°C.

Immunoprecipitations – For endogenous STAT5b immunoprecipitations from EGF-treated cells, BT-549 cells were serum starved overnight and treated with media alone or 100ng/ml recombinant human EGF (rhEGF) (Invitrogen) for 15 minutes at 37°C. For immunoprecipitations of exogenously expressed proteins, BT-549 cells were transiently transfected with HA-tagged wild-type, Y699F-, or R618K-STAT5b using Amaxa nucleofection as described above. Forty-eight hours post-transfection, cells were plated on FN-coated dishes (2µg/ml) for 1 hour at 37°C, and 1M pervandate was added for the last 15 minutes. For immunoprecipitation of endogenous proteins, lysates were made either from cells growing under normal conditions, cells placed in suspension for 1 hour, or cells re-plated on FN-coated dishes (2µg/ml) for up to 1 hour. To obtain whole-cell lysates, cells were lysed in RIPA lysis buffer [150mM NaCl, 50mM Tris, pH 7.4, 1% deoxycholate, 1% Triton X-100, 5mM EDTA] containing protease inhibitor cocktail (Calbiochem, San Diego, CA) and sodium orthovanadate (Sigma, St. Louis, MO). For immunoprecipitations, lysates were incubated with HA specific monoclonal antibody (University of Virginia Hybridoma Facility, Charlottesville, VA) or STAT1 or STAT5b specific polyclonal antibodies developed by our lab (Kloth et al., 2002; Silva

et al., 1994) overnight at 4°C. Protein A or G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added, and lysates were incubated for an additional 1 hour at 4°C. Agarose pellets were washed three times in lysis buffer, and bound proteins were removed by boiling in 2x Laemmli buffer containing β-mercaptoethanol or 20mM dithiotreitol (DTT) for 5 minutes at 100°C.

Immunoblotting – Whole cell lysates or immunoprecipitated proteins were separated on 7.5% or 12.5% polyacrylamide gels and transferred to nitrocellulose (Pall Corporation, Pensacola, FL). Membranes were blocked and incubated with primary antibodies in TBS-T [150mM NaCl, 0.1% Tween 20, 50mM Tris (pH 8.0)] containing 5% nonfat dry milk or 3% BSA. STAT5a, STAT5b, and STAT1 specific polyclonal antibodies were developed by our lab as previously described (Kloth et al., 2002; Silva et al., 1994). STAT3 polyclonal antibody and β-actin monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-HA monoclonal antibody was obtained from the University of Virginia hybridoma facility (Charlottesville, VA). The anti-STAT5a/b monoclonal antibody was purchased from BD Transduction Labs (BD Biosciences). The anti-phospho-Y699 STAT5b antibody was generated in our lab in conjunction with Aves Laboratory (Tigard, OR) (Fox et al., 2008). Monoclonal anti-c-Src antibody was obtained from the laboratory of Dr. J. Thomas Parsons (University of Virginia) (Parsons et al., 1983). Polyclonal anti-p130Cas antibody was obtained from the laboratory of Dr. Amy Bouton (University of Virginia) (Bouton and Burnham, 1997). Monoclonal anti-HA antibody was obtained from the University of Virginia hybridoma facility (Charlottesville, VA). Polyclonal anti-CXCR4, polyclonal anti-myosin light chain (MLC), polyclonal tubulin, and monoclonal anti-β-actin antibodies were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). Monoclonal anti-focal adhesion kinase (FAK) antibody was obtained from Upstate (Millipore, Billerica, MA). Polyclonal anti-phosphotyrosine-FAK (Y397) and monoclonal anti-talin antibodies were purchased from Sigma. Polyclonal anti-phosphoserine-MEK (S298) and polyclonal anti-phosphotyrosine-c-Src (Y416) antibodies were purchased from Biosource (Invitrogen, Gaithersburg, MD). Monoclonal anti-paxillin antibody was obtained from BD Transduction Labs (BD Biosciences). Secondary antibodies were applied in TBS-T and were HRP-conjugated sheep anti-mouse or donkey anti-rabbit (GE Healthcare). The enhanced chemiluminescence detection kit (GE Healthcare) was used to detect antibody binding. Acrylamide was from Bio-Rad (Hercules, CA), pre-stained molecular weight standards were from Sigma, and all other reagents were of reagent or molecular biological grade from Sigma.

Wound Healing – Cell were plated on FN-coated (5µg/ml) 35mm Bioptechs delta T dishes (Thermo Fisher Scientific). Confluent cell monolayers were wounded using a 20µl pipette tip, and cells migrating into the wound were filmed at 37^{o}C by time lapse microscopy using a Nikon TE200 inverted microscope with a 20x differential interference contrast objective and a Bioptechs heated stage. Images were taken with Hamamatsu Orca camera every 5 minutes for 6 hours and collected with Openlab software (Improvision, Lexington, MA). Movies were analyzed using Image J Manual Tracking software (National Institutes of Health, Bethesda, MD). Single cell nuclei were tracked over time and migratory speed was calculated by dividing the length of the migration path by the total movie time. Directional persistence (D/T) was determined as the net displacement (D) divided by the total length of the migration path (T). For Golgi re-orientation experiments, wounds were allowed to heal for 6 hours, after which time they

were fixed in 4% paraformaldehyde for 20 minutes at 25°C, permeabilized with 0.25% Triton X-100 for 5 minutes at 25°C, and blocked in 20% goat serum/2% BSA/PBS for 30 minutes at 25°C. Cells were then incubated with rabbit monoclonal anti-GM130 Golgi antibody (Epitomics, Burlingame, CA) for 1 hr at 25°C followed by anti-rabbit FITC (Molecular Probes, Eugene, OR) for 1 hr at 25°C. Cells were visualized with a fluorescent Leica microscope (Deerfield, IL) and images were taken with OpenLab software (Improvision) using a 20x objective.

Total Internal Reflection Fluorescence (TIRF) Microscopy – Cells were transfected with siRNA oligonucleotides, mKO-paxillin, and GFP-actin using Amaxa nucleofection as described above. Seventy-two hours post-transfection, cells were plated on FN (2μg/ml) and after 20-30 minutes of spreading, TIRF images were acquired using an inverted microscope, 60X objective (Olympus, model IX70). GFP was excited using the 488nm laser line of an Ar ion laser, and mKO was excited using the 543nm laser line of a He-Ne laser (Mells Griot, Carlsbad, CA). Time-lapse images were taken every 3 seconds for 5 minutes with a charge-coupled device camera (QImaging, Surrey, BC, Canada) and analyzed using MetaMorph (MDS Analytical Technologies, Mississauga, Ontario, Canada). Protrusion rates were determined using ImageJ Kymograph software (National Institutes of Health), and calculated as the length of the protrusion divided by the total time of the movie.

Key Research Accomplishments

- Construction of wild-type and mutant STAT5b plasmids immune to siRNA knockdown and optimization of knockdown-rescue experiments
- STAT5b, STAT5a, STAT1, and STAT3 were specifically and efficiently knocked down in breast cancer cells which express all four STAT family members
- Knockdown of STAT5b or STAT1 inhibited migration of breast cancer cells to serum, whereas knockdown of STAT5a or STAT3 had no effect on migration
- Knockdown of STAT5b inhibited migration of breast cancer cells to the serum component fibronectin
- Knockdown of STAT5b did not alter viability, adherence, or cell cycle distribution of breast cancer cells over the time course of the experiments
- Loss of migration upon STAT5b knockdown was rescued by re-introduction of either wild-type or a transcriptionally inactive Y699F-STAT5b mutant, but not an SH2 domain-defective R618K STAT5b mutant
- Knockdown of STAT5b correlated with increased number and contractility of protrusions during attachment to, and spreading on, fibronectin
- ***The data presented here identifies a novel, SH2-dependent function of STAT5b in regulating beta1 integrin-mediated migration of highly aggressive breast cancer cells***

Reportable Outcomes

Publications

- **Bernaciak TM**, Zareno J, Parsons JT, Silva CM (2009). A Novel Role for STAT5b in Beta1-Integrin-Mediated Human Breast Cancer Cell Migration. *Breast Cancer Research* 11(4):R52.
- Fox EM, **Bernaciak TM**, Wen J, Weaver AM, Shupnik MA, Silva CM (2008). STAT5b, c-Src, and EGFR Signaling Play Integral Roles in Estrogen-Stimulated Proliferation of ER-Positive Breast Cancer Cells. *Molecular Endocrinology* 22(8):1781-96.

Oral Presentations

- 25th Annual American Cancer Society Seminar of Cancer Researchers in Virginia (Richmond, VA); November 2007

  "Role of Signal Transducer and Activator of Transcription 5b (STAT5b) in Breast Cancer Cell Migration"

Poster Presentations

- Annual Meeting of the Endocrine Society (Washington, D.C.); June 2009

  P2-137: A Novel Role for Signal Transducer and Activator of Transcription 5b (STAT5b) in Beta1 Integrin-Mediated Breast Cancer Cell Migration.

  Presidential Poster Award

- University of Virginia, Department of Microbiology, Annual Retreat (Barboursville, VA); October 2008

  Role of Signal Transducer and Activator of Transcription 5b (STAT5b) in Breast Cancer Cell Migration
- Frontiers in Cell Migration Conference (Bethesda, MD); September 2008

  PII-4: Role of Signal Transducer and Activator of Transcription 5b (STAT5b) in Breast Cancer Cell Migration
- Department of Defense Breast Cancer Research Program Era of Hope Meeting (Baltimore, MD); June 2008

  P36-12: Role of STAT5b in Breast Cancer Cell Migration
- Annual Meeting of the Endocrine Society (Toronto, ON); June 2007

  P2-600: Role of Signal Transducer and Activator of Transcription 5b (STAT5b) in Breast Cancer Cell Migration and Invasion

Conclusions

Breast cancer is the second most common cancer in American women. Despite improvements in detection and the development of new treatment strategies, the American Cancer Society estimates that over 180,000 new cases of breast cancer will be diagnosed, and over 40,000 women will die from breast cancer this year alone. Because many cancers arise from dysregulation of signaling pathways found in normal cells, one of the difficulties in treating cancers is identifying cancer-specific therapeutic targets.

Breast tumors are routinely tested for the overexpression of the hormone receptors for estrogen (ER) and progesterone (PR), as well as human epidermal growth factor receptor 2 (HER2). Targeted therapies for ER (Tamoxifen) and HER2 (Herceptin) are available to treat cancers overexpressing these receptors. However, not all patients respond to these drugs, and those that do often develop resistance. Furthermore, approximately 15% to 20% of breast tumors are negative for all three receptors (termed "triple-negative") and thus not eligible for these therapies.

EGFR-overexpressing tumors account for up to 48% of breast tumors, a subset of which fall into the triple-negative classification. Breast tumors which express elevated levels of EGFR are generally more aggressive, are associated with increased metastasis, and correlate with poor clinical outcome (Nielsen et al., 2004). The poor clinical outcome is reflective of a lack of effective treatments. At this time, there are no successful therapies for preventing or treating metastatic breast cancer. For this reason, it is of utmost importance that we identify therapeutic targets for EGFR-overexpressing tumors.

Current therapies designed to target the EGFR have not been as successful as anticipated. This lack of success is due in part to the ability of cancer cells to upregulate alternative signaling

pathways to promote growth and tumor progression. Many tumorigenic signaling pathways converge on common nuclear transcription factors and therefore, targeting these downstream proteins may be more effective (Darnell, 2002). One such group of transcription factors is the signal transducer and activator of transcription (STAT) family. STAT proteins regulate gene transcription in both normal and cancer cells to promote proliferation, survival, angiogenesis, migration, and invasion downstream of both cytokine and growth factor receptors (Bromberg, 2002). STAT5b, a member of this family, is overexpressed and constitutively activated in a number of cancers, including breast cancer (Bowman et al., 2000; Haura et al., 2005; Turkson and Jove, 2000). Prior to this work, the contribution of STAT5b to breast cancer focused on its growth promoting abilities downstream of EGFR and ER signaling (Boerner et al., 2004; Fox et al., 2008; Kloth et al., 2002; Kloth et al., 2003; Riggins et al., 2006; Weaver and Silva, 2006). This thesis centers on evaluating the importance of STAT5b in breast cancer motility with the goal of further expounding on the migratory mechanisms contributing to metastasis.

Knockdown of STAT5b inhibits migration of two highly aggressive, EGFR-overexpressing breast cancer cell lines, BT-549 and MDA-MB-231, to serum. Despite high expression of EGFR, these cell lines do not migrate robustly to EGF (Figure 8). They do, however, migrate very well to the extracellular matrix component fibronectin (Figure 16). Migration resulting from attachment to fibronectin is mediated through β1 integrin receptors. Pre-treatment of cells with a β1 integrin blocking antibody greatly suppresses migration to serum (Figure 16), indicating that fibronectin is a major component of serum responsible for stimulating breast cancer cell migration. Knockdown of STAT5b inhibits migration to BT-549 and MDA-MB-231 cells to fibronectin (Figure 16), illustrating its importance for β1 integrin-mediated migration.

Phosphorylation of Y699 on STAT5b is not necessary for promoting migration, nor is the C-terminal transactivation domain (Figure 18). In contrast, the SH2 domain is critical, as evidenced by the lack of rescue of an SH2 domain defective STAT5b mutant (R618K) following STAT5b knockdown (Figure 18). As a whole, these data support an SH2-dependent cytoplasmic function of STAT5b in regulating breast cancer cell migration. The long-standing paradigm for STAT signaling is that of nuclear regulation of gene transcription. However, in the last few years, evidence for cytoplasmic activity of transcription factors has come to light. Perhaps the most well known example is that of the tumor suppressor p53. p53 was initially believed to induce apoptosis through transcriptional regulation of apoptotic genes such as Bax (Miyashita and Reed, 1995). More recently it has been discovered that p53 also induces apoptosis in the cytoplasm through association with Bcl-x_L, promoting cytochrome c release from the mitochondria (Mihara et al., 2003). Additionally, the multifunctional transcription factor TFII-I can associate with PLCγ in the cytoplasm to regulate calcium influx (Caraveo et al., 2006). More relevant to our studies are the novel cytoplasmic roles of STAT1 in bone remodeling and STAT3 in microtubule dynamics. In osteoblasts, cytoplasmic unphosphorylated STAT1 associates with the transcription factor Runx2, sequestering it in the cytoplasm, thereby inhibiting osteoblast differentiation (Kim et al., 2003). STAT3 regulates microtubule dynamics in the cytoplasm through associating with and inhibiting the microtubule depolymerizing protein stathmin (Ng et al., 2006). Both of these cytoplasmic STAT functions are independent of tyrosine phosphorylation. Given this data, it is not surprising that other STATs could actively function in the cytoplasm in their unphosphorylated state, rather than simply being latent proteins.

Indeed, our work supports an active cytoplasmic role of unphosphorylated STAT5b in breast cancer cell migration in multiple migratory processes. Loss of STAT5b leads to a polarity defect which impedes directional movement (Figures 20, 21). During spreading on fibronectin, STAT5b knockdown cells take on a remarkable phenotype distinguished by the formation of multiple, unstable protrusions (Figure 22). There is no defect in initial attachment and once attached, protrusions extend rapidly (Figures 22, 23). However, these protrusions are highly dynamic and over time they contract back into the cell. This phenotype is indicative of disrupted equilibrium between Rho family GTPases. Rac is predominantly localized at the leading edge with Rho in the tail. Normal ratios of Rho and Rac lead to Rac-mediated formation and spreading of broad, protrusive lamellipodia in the front of the cell followed by Rho-mediated tail retraction, overall resulting in directional migration toward a stimulus (Burridge and Doughman, 2006). Increased Rho activity in the front of the cell would disrupt this equilibrium and could account for the multiple protrusions and increased contraction at the front of the cell seen with STAT5b knockdown. This disrupted equilibrium could be a consequence of either mislocalization of Rho and Rac or alterations in their activity. Based on the data presented in this dissertation, we postulate that unphosphorylated STAT5b influences multiple aspects of migration (polarity, protrusion, and contractility) through regulation of cytoplasmic GTPase signaling.

Although we have not determined the full mechanism by which STAT5b promotes migration, this body of work provides a substantial starting point. Rho family signaling downstream of integrin engagement is well defined (Figure 4). To expand on the mechanism, expression and activation of Rho and Rac could be compared in control and STAT5b knockdown cells to determine where in the pathway STAT5b is functioning. Our initial attempts to examine

Rac activity were unsuccessful (*data not shown*), but we are confident that with proper optimization of assay conditions, we will be able to efficiently isolate Rac from both BT-549 and MDA-MB-231 breast cancer cell lines. Additionally, differential association of wild-type and R618K-STAT5b with cytoplasmic proteins following integrin engagement should be explored. Preliminary studies employing transient transfection of HA-tagged STAT5b constructs showed association of wild-type, Y699F-, and R618K-STAT5b with endogenous STAT5b (*data not shown*). This result is not unexpected given the high affinity of STAT5b monomers for each other. To help circumvent wild-type STAT5b homodimers, these experiments could be performed in the presence of STAT5b siRNA, thus eliminating endogenous STAT5b and enabling lower affinity interactions to be observed.

Determining where STAT5b is localized in migrating cells would be enlightening. Unfortunately, this has been technically difficult. With the antibodies currently available, we have not been able to successfully stain for endogenous STAT5b without significant background (*data not shown*). Although visualizing endogenous STAT5b would be ideal, as an alternative, we have attempted to track GFP-tagged exogenous STAT5b in live spreading cells. Despite varying concentrations of transfected plasmid, we observe high levels of GFP-STAT5b throughout the cell, making tracking difficult (*data not shown*). To circumvent this, GFP-STAT5b could be placed into a low-expression vector.

Furthermore, the role of STAT5b in *in vivo* tumor formation and metastasis must be addressed. Based on our *in vitro* studies, knockdown of STAT5b inhibits migration of breast cancer cells, but overexpression of STAT5b does not enhance migration (Figures 9, 12). Therefore, we would examine the effect of loss of STAT5b on *in vivo* metastasis. To do so, STAT5b would be stably knocked down using lentiviral transduction and control or knockdown

cells would be orthotopically implanted into mammary fat pads of female athymic nude mice. The ideal cell line for these studies is mouse mammary 4T1 cells because they undergo rapid, spontaneous metastasis to the lung and liver, common sites of human breast cancer metastasis (Aslakson and Miller, 1992). However, it must first be established if these cells express STAT5b and if so, whether or not STAT5b can be stably knocked down in these without adverse effects on viability. As an alternative, MDA-MB-231 human breast cancer cells, known to express STAT5b, could be used. In these studies, the effect of STAT5b knockdown, compared to control knockdown, would be investigated. As there are many signaling pathways involved in metastasis, a complete abrogation of metastasis with STAT5b knockdown is not expected. Rather, it is anticipated that the number of metastases, size, and/or time to metastasis would be decreased.

STAT proteins are involved in many processes of tumorigenesis (proliferation, survival, angiogenesis, migration, and invasion), and mediate signaling of kinases upregulated in cancer, making them potential targets for cancer therapies (Bowman et al., 2000; Bromberg and Darnell, 2000; Silva and Shupnik, 2007; Turkson and Jove, 2000). Targeting STAT proteins is challenging because they do not have enzymatic activity or bind ligands. However, there are multiple steps required for STAT activation that can be exploited, including peptides that interfere with receptor association or "decoy" oligonucleotides that inhibit DNA binding (Germain and Frank, 2007). To disrupt receptor association, small peptides targeting the SH2 domain of STAT3 and STAT5b have been developed (McMurray, 2006; Muller et al., 2008). Because there are countless proteins that contain SH2 domains, more thorough analysis of peptide specificity must be performed. Moreover, data on the biological effects of these inhibitors is limited and needs to be expanded. In contrast, a substantial amount of work has

been done on *in vitro* and *in vivo* efficacy of a STAT3 "decoy" oligonucleotide. A decoy is a segment of double-stranded DNA corresponding to the response element located in the promoter of a target gene, which at high enough concentrations, inhibits DNA binding (Nabel et al., 1990). Treatment of squamous cell carcinoma of the head and neck (SCCHN) cell lines with a STAT3 decoy inhibited DNA binding and suppressed proliferation (Leong et al., 2003). Furthermore, daily injection of STAT3 decoy into SCCHN xenografts suppressed STAT3 target gene expression, decreased proliferation and increased apoptosis (Xi et al., 2005). This effect was enhanced with the chemotherapeutic agent cisplatin. This work supports the use of STAT decoys as a cancer therapy. Nonetheless, given our data, and that of others, identifying transcription-independent functions of STAT proteins, it is important to devise therapies targeting both transcriptional and cytoplasmic functions.

Although our work has been done in a breast cancer model system, it is important to note that cell migration is involved in a variety of processes other than tumor metastasis. Cell migration is critical for embryonic development and lack of efficient migration can lead to death or a variety of birth defects ranging from spina bifida and improper organ development to mental retardation (Juriloff and Harris, 2000; Verrotti et al., 2009). In adults, migration is a vital step in immune response and migration defects can affect numerous processes from fighting infection to proper wound healing (Barrientos et al., 2008; Martin and Leibovich, 2005; Woodland and Kohlmeier, 2009). Therefore, STAT5b migratory signaling has the potential to affect a variety of biological processes and its role in these processes warrants further investigation.

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Appendix

Bernaciak TM, Zareno J, Parsons JT, Silva CM (2009). A Novel Role for STAT5b in Beta1-Integrin-Mediated Human Breast Cancer Cell Migration. *Breast Cancer Research* 11(4):R52.

Research article

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A novel role for signal transducer and activator of transcription 5b (STAT5b) in β_1-integrin-mediated human breast cancer cell migration

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Abstract

Introduction Signal transducer and activator of transcription (STAT) 5b is a transcription factor involved in pro-proliferative and pro-survival signaling in a number of solid tumors, including breast cancer. The contribution of STAT5b to breast cancer cell motility has not been explored. This work aims to elucidate the role of STAT5b in breast cancer cell migration.

Methods STAT5b was knocked down by using siRNA in two aggressive, highly migratory breast cancer cell lines (BT-549 and MDA-MB-231), and transwell migration assays were performed to determine the importance of STAT5b for their migration. Knockdown-rescue experiments were used to validate the specificity of STAT5b knockdown and to determine which regions/functions of STAT5b are necessary for its role in migration. Live-cell imaging of wound healing and spreading was carried out to examine cell morphology and motility after STAT5b knockdown.

Results Knockdown of STAT5b, but not STAT5a, inhibited migration of BT-549 and MDA-MB-231 breast cancer cells to serum by 60% to 80%, and inhibited migration equally over a range of serum concentrations (0.1% to 10% serum). Migratory inhibition upon STAT5b knockdown could be rescued by reintroduction of wild-type STAT5b, as well as Y699F- and dominant-negative STAT5b mutants, but not an SH2 domain defective R618K-STAT5b mutant. β_1- integrin-mediated migration of breast cancer cells to fibronectin was inhibited with STAT5b knockdown, and loss of STAT5b correlated with loss of directional migration and formation of multiple, highly contractile protrusions upon attachment to fibronectin.

Conclusions The data presented here demonstrate that STAT5b is integral to breast cancer cell migration and identify a novel, SH2-dependent function of STAT5b in regulating β_1-integrin-mediated migration of highly aggressive breast cancer cells.

Introduction

Breast cancer is the second most common cancer in American women. Despite improvements in detection and the development of new treatment strategies, the American Cancer Society estimates that more than 180,000 new cases of breast cancer will be diagnosed, and more than 40,000 women will die of breast cancer this year alone. Because many cancers arise from dysregulation of signaling pathways found in normal cells, one of the difficulties in treating cancers is identifying cancer-specific therapeutic targets. Current targeted therapies have not been as successful as anticipated. This lack of success is due in part to the ability of cancer cells to upregulate alternative signaling pathways to promote growth and tumor progression. Many tumorigenic signaling pathways converge on common nuclear transcription factors, and therefore, targeting these downstream proteins may be more effective [1].

BSA: bovine serum albumin; DMEM: Dulbecco's modified eagle's medium; DMSO: dimethyl sulfoxide; dn: dominant-negative; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; F: phenylalanine; FBS: fetal bovine serum; FN: fibronectin; GFP: green fluorescent protein; GH: growth hormone; HA: hemagglutinin; K: lysine; LPA: lysophosphatidic acid; PBS: phosphate-buffered saline; Prl: prolactin; R: arginine; RFP: red fluorescent protein; SCCHN: squamous cell carcinoma of the head and neck; SH2: Src homology domain 2; STAT: signal transducer and activator of transcription; TAD: transactivation domain; TIRF: total internal reflection fluorescence; VN: vitronectin; wt: wild type; Y: tyrosine.

One such group of transcription factors is the signal transducer and activator of transcription (STAT) family. STATs are a family of transcription factors activated by cytokines or growth factors or both. Seven members of the STAT family are known: STAT 1, 2, 3, 4, 5a, 5b, and 6. STAT proteins are latent in the cytoplasm and require phosphorylation of a conserved C-terminal tyrosine residue for activation. This allows dimerization to occur between the phosphorylated tyrosine of one STAT and the Src homology 2 (SH2) domain of another. Active dimers are translocated to the nucleus, where they bind DNA and regulate gene transcription. STAT proteins regulate transcription of genes involved in a variety of biologic processes, including proliferation, survival, and angiogenesis, all of which are involved in cancer development and progression. Thus, it is not surprising that in the last several years, a role for STATs in tumorigenesis has emerged. Activation of STAT5a and STAT5b occurs in a variety of cancers including both hematopoietic cancers and solid tumors, such as those of the breast, prostate, lung, head and neck, and brain [2,3]. STAT5a and STAT5b regulate the transcription of the pro-proliferative genes *c-myc* and *cyclin D1* and the anti-apoptotic genes *Bcl-xL* and *Pim-1*, to stimulate tumor growth and survival [4-8]. In addition, STAT5b has been implicated in prostate cancer cell invasion [9].

To date, most of the work examining STAT5b in breast cancer has focused on its pro-proliferative function, and its role in breast cancer cell migration has not been examined. Importantly, a recent study investigating the effects of STAT5a on breast cancer cell migration and invasion showed that prolactin (Prl)-induced activation of STAT5a inhibited migration and invasion of BT-20 and T-47D human breast cancer cells [10]. STAT5a and STAT5b, although highly homologous, are encoded by two separate genes and function independently in mammary gland development. STAT5a is necessary for lobuloalveolar outgrowth and lactation mediated by Prl signaling, whereas STAT5b is vital for establishing growth hormone (GH)-directed sexual dimorphism [11,12].

Given this background, we sought to investigate the potential role of STAT5b, specifically, in the migration of two highly aggressive, highly migratory breast cancer cell lines. We found that STAT5b knockdown inhibited serum- and fibronectin-stimulated migration of both BT-549 and MDA-MB-231 human breast cancer cell lines in a transwell assay. This inhibition was rescued by co-expression of wild-type, Y699F-, and dominant-negative STAT5b but not STAT5b containing a mutation in the SH2 domain. With real-time imaging, we showed that knockdown of STAT5b resulted in decreased directional migration and the formation of multiple protrusions, giving rise to an overall reduction in motility. These results establish, for the first time, an important SH2-dependent function of STAT5b in aggressive breast cancer, further defining its role in tumorigenesis and supporting its potential as a therapeutic target for the treatment of breast cancer.

Materials and methods

Cell culture

BT-549 and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were passaged twice per week and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All tissue-culture reagents were purchased from Invitrogen (Gaithersburg, MD).

siRNA Transfection

BT-549 and MDA-MB-231 cells were transfected with siGENOME SMARTpool siRNA targeting human STAT5b or individual custom oligonucleotides specific for STAT5a or STAT5b (siGENOME STAT5b SMARTpool duplex #3), or luciferase duplex control, all purchased from Dharmacon (Lafayette, CO). Transfections were performed by using either Oligofectamine (Invitrogen) or Amaxa nucleofection (Amaxa/Lonza, Walkersville, MD), by using solution T and program X-013 (MDA-MB-231 cells) or A-023 (BT-549 cells) as per manufacturers' instructions. For knockdown-rescue experiments, cells were transfected simultaneously with siSTAT5b SMARTpool duplex #3 and HA-tagged wild-type-, Y699F-, dominant-negative-, or R618K-STAT5b engineered to be immune to knockdown by introduction of four silent point mutations in the siRNA target sequence. These point mutations were introduced by using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), and constructs were sequenced to verify mutations.

Immunoblotting

Cells were lysed in RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris, pH 7.4, 1% deoxycholate, 1% Triton X-100, 5 mmol/L EDTA) containing protease inhibitor cocktail (Calbiochem, San Diego, CA) and sodium orthovanadate (Sigma, St. Louis, MO), and boiled in 2× Laemmli buffer containing β-mercaptoethanol or 20 mmol/L dithiothreitol for 5 minutes at 100°C. Protein lysates were separated on 7.5% or 12.5% polyacrylamide gels and transferred to nitrocellulose (Pall Corporation, Pensacola, FL). Membranes were blocked and incubated with primary antibodies in TBST (150 mmol/L NaCl, 0.1% Tween 20, 50 mmol/L Tris, pH 8.0) containing 5% nonfat dry milk or 3% BSA. STAT5a- and STAT5b-specific polyclonal antibodies were developed by our laboratory, as previously described [13]. Monoclonal anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HA monoclonal antibody was obtained from the University of Virginia hybridoma facility. Secondary antibodies were applied in TBST and were HRP-conjugated sheep anti-mouse or donkey anti-rabbit (GE Healthcare, Piscataway, NJ). The enhanced chemiluminescence detection kit (GE Healthcare) was used to detect antibody binding. Acrylamide was from Bio-Rad (Hercules, CA), prestained molecular weight standards were from Sigma, and all other reagents were of reagent or molecular biologic grade from Sigma.

Transwell migration assays

BT-549 and MDA-MB-231 cells were transfected with siRNA, as described earlier. Seventy-two hours after transfection, 5×10^4 BT-549 cells or 1×10^5 MDA-MB-231 cells were plated in serum-free (DMEM/0.1% BSA) media into the upper chambers of BD BioCoat Control Chambers (BD Biosciences, San Jose, CA), and DMEM containing 0 to 10% FBS was placed in the lower chamber. For β_1-integrin blocking experiments, cells were pretreated for 1 hour with DMSO (vehicle control) or 10 μg/ml monoclonal anti-human β_1-integrin antibody (R&D Systems, Minneapolis, MN), and treatment was left on for the duration of the assay. For migration to extracellular matrix components, the undersides of filters were coated with 0 to10 μg/ml human plasma fibronectin (FN) (BD Biosciences) or recombinant human vitronectin (VN) (R&D Systems) overnight at 4°C, as indicated, and DMEM/0.1% BSA was used in both the upper and lower chambers. Plates were incubated at 37°C, and migration was allowed to proceed for 3 to 6 hours. After this time, nonmigratory cells in the upper chambers were removed with cotton swabs, and the remaining cells were stained with 0.1% crystal violet (Sigma) in 20% ethanol. Cells were counted by using a Zeiss Invertoskop light microscope. Four fields were counted on each of two filters. Results are expressed as average cells per field or relative migration (compared with control).

Wound healing

Cell were plated on FN-coated (5 μg/ml) 35-mm Bioptechs delta T dishes (Fisher Scientific, Pittsburgh, PA). Confluent cell monolayers were wounded by using a 20-μl pipette tip, and cells migrating into the wound were filmed at 37°C with time-lapse microscopy by using a Nikon TE200 inverted microscope with a 20× differential interference contrast objective and a Bioptechs heated stage. Images were taken with a Hamamatsu Orca camera every 5 minutes for 6 hours and collected with Openlab software (Improvision, Lexington, MA). Movies were analyzed by using Image J Manual Tracking software (National Institutes of Health, Bethesda, MD). Single cell nuclei were tracked over time, and migratory speed was calculated by dividing the length of the migration path by the total movie time. Directional persistence was determined as the net displacement divided by the total length of the migration path.

Total internal reflection fluorescence (TIRF) microscopy

Cells were transfected with siRNA oligonucleotides, mKO-paxillin, and GFP-speckle-actin by using Amaxa nucleofection, as described earlier. Seventy-two hours after transfection, cells were plated on FN (2 μg/ml), and after 20 to 30 minutes of spreading, TIRF images were acquired by using an inverted microscope, 60× objective (Olympus, model IX70). GFP was excited by using the 488-nm laser line of an Ar ion laser, and RFP was excited by using the 543-nm laser line of an He-Ne laser (Mells Griot, Carlsbad, CA). Time-lapse images were taken every 3 seconds for 5 minutes with a charge-coupled device camera (QImaging, Surrey, BC, Canada) and analyzed by using MetaMorph (MDS Analytical Technologies, Mississauga, Ontario, Canada). Protrusion rates were determined by using ImageJ Kymograph software (National Institutes of Health) and calculated as the length of the protrusion divided by the total time of the movie.

Results

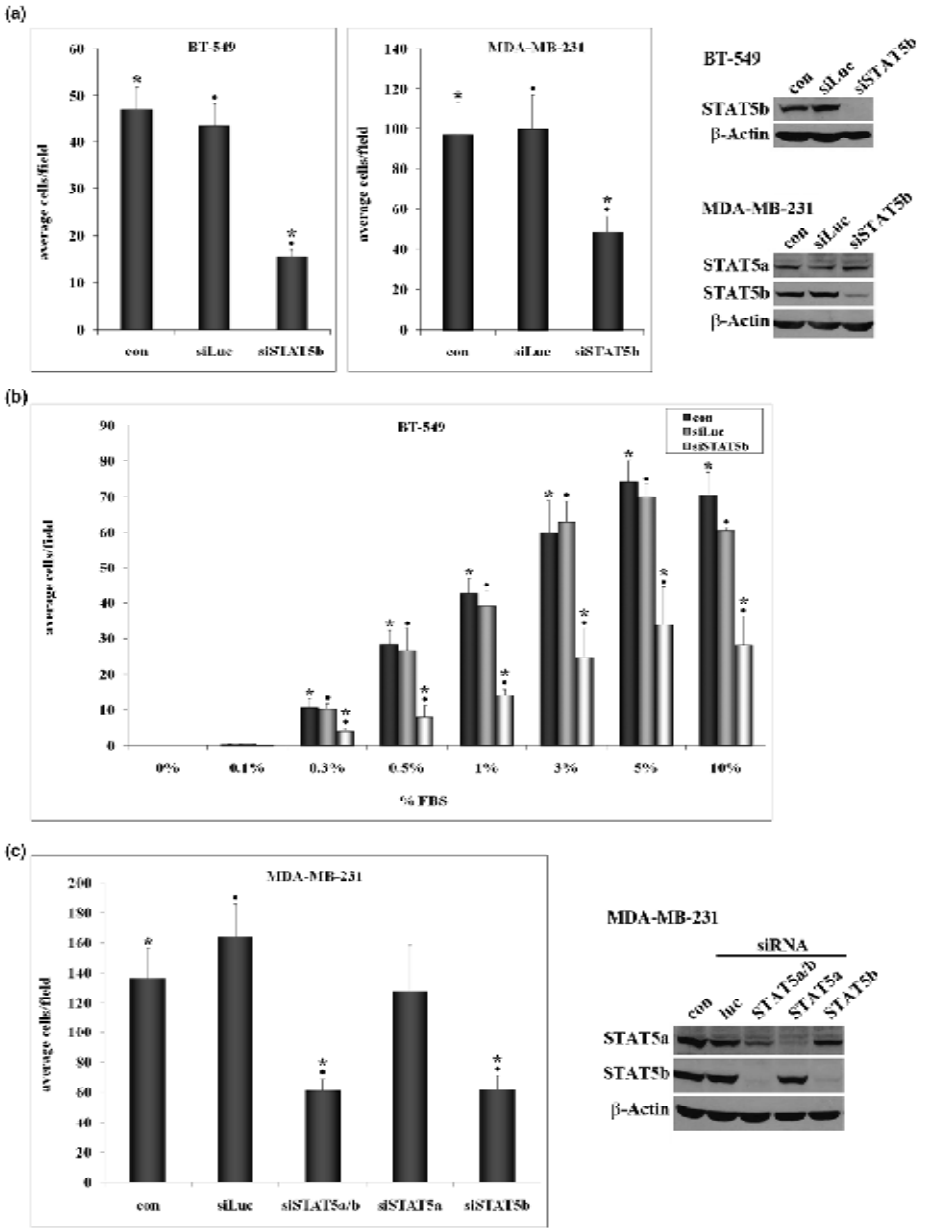
STAT5b knockdown inhibits breast cancer cell migration

To determine the importance of STAT5b for breast cancer cell migration, we used siRNA transfection to knock down STAT5b in the highly migratory BT-549 breast cancer cell line. Knockdown of STAT5b to virtually undetectable levels in these cells inhibited their migration to serum by approximately 70% (Figure 1A). To ensure that this effect was not unique to one cell line, we tested the effect of STAT5b siRNA knockdown on migration of MDA-MB-231 cells, another highly aggressive, migratory breast cancer cell line. In these cells, knockdown of STAT5b also inhibited migration to serum, by approximately 50% (Figure 1A).

It is well established that STAT5b promotes cell-cycle progression and survival of breast cancer cells [14-19]. To determine whether knockdown of STAT5b affected proliferation or survival under the conditions of our migration experiments, we performed trypan blue assays on knockdown cells 72 hours after transfection. In both BT-549 and MDA-MB-231 cells, knockdown of STAT5b did not significantly alter the total number of adherent cells or the viability of adherent cells over a 6-hour period, which is the maximum length of our transwell migration assays (data not shown). Thus, we conclude that the inhibition of migration after knockdown of STAT5b is not a result of secondary effects on adherence, viability, or survival.

By using BT-549 cells, we determined the optimal migration conditions for trans-well assays to be the migration to 1% serum over a 3-hour period. Because these cells require a low dose of serum to migrate, we used them to determine whether increasing concentrations of serum could overcome the effect of STAT5b knockdown. As seen in Figure 1B, migration to serum was dose dependent, with 3% to 10% serum being optimal. Knockdown of STAT5b significantly inhibited migration of BT-549 cells by 60% to 80% at all serum concentrations. Similar results were obtained in the MDA-MB-231 cells (data not shown). Thus, increasing the concentration of serum components did not overcome the effect of STAT5b knockdown on inhibiting migration. However, migration was not completely abrogated after knockdown of STAT5b in either cell line. The cells retained their capacity to migrate at a similar "basal" level across all serum concentrations tested. This indicates that both STAT5b-dependent and STAT5b-independent pathways may be responsible for migration of these cells to serum.

Figure 1



STAT5b knockdown inhibits breast cancer cell migration. **(a)** BT-549 or MDA-MB-231 breast cancer cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc), siSTAT5b SMARTpool (BT-549 cells), or siSTAT5b SMARTpool duplex #3 (MDA-MB-231 cells). Seventy-two hours after transfection, cells were plated in serum-free media in trans-well chambers. Media containing 1% fetal bovine serum (FBS; BT-549) or 10% FBS (MDA-MB-231) were placed in the lower chambers. After 3 hours (BT-549) or 6 hours (MDA-MB-231), cells were fixed, stained with crystal violet, and the number of migratory cells was counted. Results are graphed as the average number of migratory cells per field ± SEM. One-way ANOVA with Tukey's post-test was used to determine statistical significance ($P < 0.05$) between the following: BT-549: con and siSTAT5b (*), siLuc and siSTAT5b (black circles); n = 5. MDA-MB-231: con and siSTAT5b (*), siLuc and siSTAT5b (black circles); n = 4. (b) BT-549 breast cancer cells were transfected and plated in serum-free media in trans-well chambers for 3 hours. Media containing varying concentrations of FBS (0.1% to 10%) were placed in the lower chambers. One-way ANOVA with Tukey's post-test was used to determine statistical significance ($P < 0.05$) between the following: con and siSTAT5b (*), siLuc and siSTAT5b (black circles); n = 3. **(c)** MDA-MB-231 cells were transfected with no siRNA (con), control siRNA for luciferase (siLuc), or siRNA specific to STAT5a/b (siSTAT5b siGENOME SMARTpool), STAT5a (siSTAT5a custom oligonucleotide), or STAT5b (siSTAT5b siGENOME SMARTpool oligonucleotide 3), and trans-well migration assays were performed as described. One-way ANOVA with Tukey's post-test was used to determine statistical significance ($P < 0.05$) between the following: con and siSTAT5a/b (*), con and siSTAT5b (*), siLuc and siSTAT5a/b (black circles), siLuc and siSTAT5b (black circles); n ≥ 5. (a and c) Whole-cell lysates from siRNA-transfected cells were immunoblotted with antibodies specific for STAT5a, STAT5b, and β-actin as a loading control.

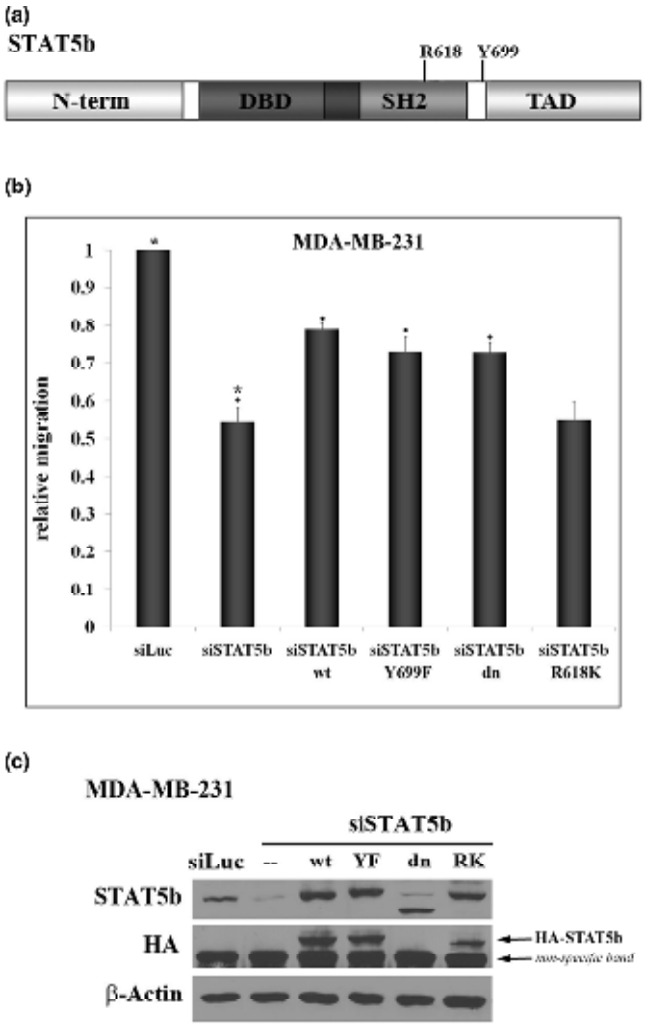
Because STAT5a has been implicated in breast cancer migration [10], we investigated the role of STAT5a on migration in our model system. We chose to use the MDA-MB-231 breast cancer cell line because they are highly migratory and express both STAT5a and STAT5b proteins, whereas BT-549 breast cancer cells express only STAT5b (data not shown). To target STAT5b specifically in the MDA-MB-231 cells, we used one of the individual oligonucleotides from the SMARTpool, which had no effect on STAT5a levels (Figure 1C, lane *5*). The total SMARTpool, previously used in the BT-549 cells, was used to determine the effect of dual knockdown of STAT5a and STAT5b in our MDA-MB-231 model system. Whereas knockdown of STAT5b inhibited migration of MDA-MB-231 cells by greater than 50%, knockdown of STAT5a had no significant effect on migration of these cells (Figure 1C). Furthermore, knockdown of STAT5a in combination with STAT5b did not enhance the inhibition of migration due to knockdown of STAT5b alone. Therefore, STAT5b is necessary for optimal migration of highly aggressive breast cancer cells, whereas expression of STAT5a is not required and cannot compensate for loss of STAT5b.

To eliminate the possibility of off-target effects of STAT5b knockdown, we performed knockdown-rescue experiments. MDA-MB-231 cells were simultaneously transfected with STAT5b-specific siRNA and hemagglutinin (HA)-tagged wild-type STAT5b that is resistant to knockdown. Consistent with previous experiments, knockdown of STAT5b inhibited migration of MDA-MB-231 cells by approximately 60%. Re-introduction of wild-type STAT5b restored migration to approximately 76% of control levels (Figure 2B), confirming that the inhibition of migration with STAT5b knockdown is due to a direct effect of STAT5b on migratory pathways. In these experiments, transfection efficiency of siRNA was close to 100%, whereas transfection efficiency of rescue constructs was approximately 65% to 75% (data not shown).

Expression of wild-type, Y699F-, or dominant-negative STAT5b rescues migration, but expression of R618K-STAT5b does not

Additional knockdown-rescue experiments were performed to identify the functional domains required for rescue of the siRNA phenotype. We introduced Y699F-, dominant-negative (dn)-, and R618K-STAT5b mutants to test the requirement of transcriptional activity and active Y699-SH2 STAT5b dimers for promoting migration. Y699F-STAT5b cannot be phosphorylated on the conserved tyrosine residue Y699, and dn-STAT5b lacks the C-terminal transactivation domain, rendering these constructs transcriptionally inactive [14,17,20]. R618K-STAT5b has a mutation in the conserved arginine required for SH2 domains to bind phosphorylated tyrosines, interfering with the ability of this mutant to form active dimers with Y699-phosphorylated STAT5b [21]. In migration assays, re-introduction of Y699F- and dn- STAT5b into MDA-MB-231 cells rescued migration to the same level as did rescue with

Figure 2



Expression of wild-type, Y699F-, or dominant-negative STAT5b rescues migration, but expression of R618K-STAT5b does not. **(a)** Domain structure of the STAT5b protein depicting the amino terminus (N-term), DNA-binding domain (DBD), Src homology 2 domain (SH2), transactivation domain (TAD), and the location of the conserved tyrosine residue, Y699, and arginine residue, R618. **(b)** MDA-MB-231 breast cancer cells were transfected with control siRNA to luciferase (siLuc) or siRNA specific to STAT5b (siSTAT5b siGENOME SMARTpool oligonucleotide #3) alone or in the presence of HA-tagged wild-type (wt-STAT5b), Y699F (YF-STAT5b), dominant-negative (dn-STAT5b), or R618K (RK-STAT5b) STAT5b constructs engineered to be immune to siRNA knockdown. Seventy-two hours after transfection, trans-well assays were performed for 6 hours, as described in Figure 1. Results are graphed as relative migration, compared with siLuc control. One-way ANOVA with Tukey's post-test was used to determine statistical significance ($p < 0.05$) between the following: siLuc and siSTAT5b (*), siSTAT5b and siSTAT5b + wt-STAT5b (black circles), siSTAT5b and siSTAT5b + YF-STAT5b (black circles), siSTAT5b and siSTAT5b + dn-STAT5b (black circles); $n \geq 4$. Comparison of siSTAT5b and siSTAT5b + RK-STAT5b was not statistically significant. Whole-cell lysates from transfected cells were immunoblotted with antibodies specific for STAT5b, HA, or β-actin as a loading control.

wild-type STAT5b. In contrast, the R618K-STAT5b mutant could not rescue migration (Figure 2B). Taken together, these data indicate that STAT5b-mediated transcription is not necessary for regulating migration. Moreover, the SH2 domain has an integral role, separate from that of active dimer formation, in promoting migration.

STAT5b knockdown inhibits β_1-integrin-mediated migration to FN

We sought to determine the serum component responsible for migration of breast cancer cells as a means of gaining insight into those migratory signaling pathways to which STAT5b contributes. Despite previously published work, we did not observe migration of BT-549 or MDA-MB-231 cells to the chemokine stromal cell-derived factor-1β (SDF-1β) [22]. In addition, no migration was observed to lysophosphatidic acid (LPA), a major component of serum. Interestingly, in the absence of serum, neither cell line migrated to epidermal growth factor (EGF), despite expression of the epidermal growth factor receptor (EGFR). We showed previously that EGF stimulation of breast cancer cells leads to phosphorylation of STAT5b on Y699 and subsequent transcriptional activity [13,14,16,17]. Therefore, the lack of migration to EGF is consistent with the observation that Y699 phosphorylation and active transcription is not required for the STAT5b migratory function. Additionally, we did not detect either basal or serum-induced Y699 phosphorylation in these cells, further supporting the idea that STAT5b mediates migration to serum through a separate, transcription-independent pathway.

Next, we tested migration of cells to extracellular matrix components known to be present in serum, specifically fibronectin (FN) and vitronectin (VN). Both BT-549 and MDA-MB-231 cells migrated to FN, whereas only BT-549 cells migrated significantly to VN (Figure 3A). Therefore, to compare effects on both cell lines, we used fibronectin as the migratory stimulus. Cells attach to fibronectin and mediate signaling through integrin receptors comprising β_1-integrin in combination with various α-integrins. To confirm that fibronectin was indeed a major serum component stimulating migration, we pretreated cells with a β_1-integrin blocking antibody and measured migration to serum. Inhibition of β_1-integrin receptor inhibited migration of both cell lines to serum by approximately 50% compared with the vehicle control (Figure 2B).

Nonspecific mouse IgG had no effect on migration (data not shown). Fibronectin dose curves were performed, and it was found that optimal migration of both BT-549 and MDA-MB-231 cell lines occurred at 2 to 3 μg/ml FN (data not shown). This is consistent with a study by Hayman and colleagues [23], which measured the level of FN in fetal bovine serum to be 25 μg/ml, or 2.5 μg/ml in 10% FBS/DMEM. To determine whether STAT5b is important in β_1-integrin-mediated migration, we examined the effect of STAT5b knockdown on migration to FN. Knockdown of STAT5b inhibited migration of both cell lines to FN, to the same extent that knockdown inhibited migration to serum, approximately 40% to 60% (Figure 3C). Taken together, these results demonstrate that STAT5b is integral for β_1-integrin-mediated migration of breast cancer cells to FN.

STAT5b knockdown results in multiple protrusions and negatively affects directionality during wound closure

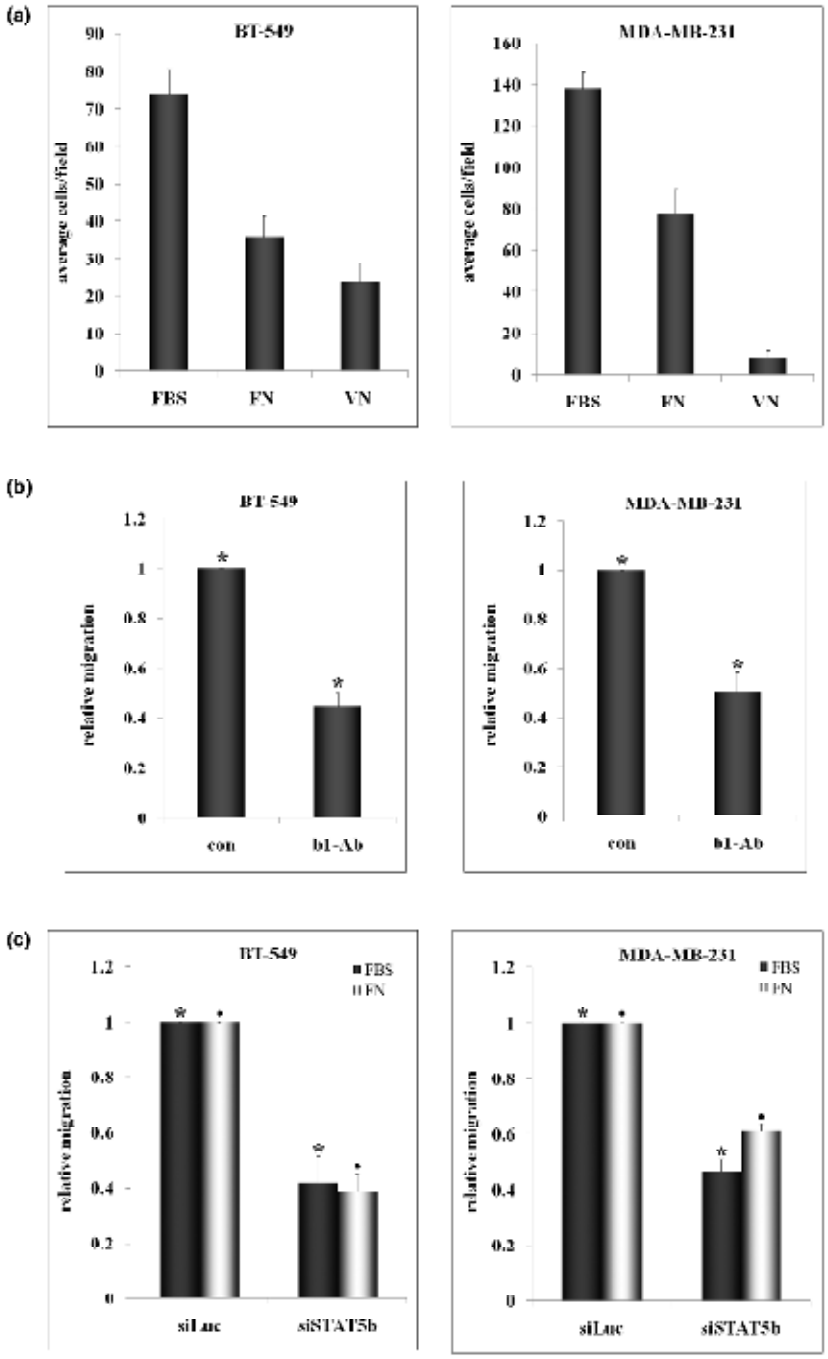
Wound-healing assays were performed to examine cell morphology and motility during wound closure of cells plated on FN. After STAT5b knockdown, wounds were allowed to close for 6 hours, and time-lapse microscopy was used to track cell movement into the wound. Figure 4A depicts the paths of cells (tracked by single cell nuclei) along the wound edge. Control cells migrated in relatively straight lines into the wound area. In contrast, STAT5b-knockdown cells showed compromised directionality in movement, migrating both horizontally and vertically. To quantitate these results, we measured migration rate and directional persistence (Figure 4B and 4C). Knockdown of STAT5b had little effect on overall migration rate; however, cells deficient in STAT5b exhibited significant loss of directional persistence. Additionally, the number of cells with Golgi localized between the nucleus and leading edge during wound closure was significantly less after STAT5b knockdown (data not shown), suggestive of a defect in cell polarity. This is consistent with findings that STAT5b-knockdown cells migrate less efficiently in transwell assays (Figure 1).

To examine focal adhesion and actin dynamics at the membrane, total internal reflection fluorescence (TIRF) microscopy was used to image cells plated on FN. STAT5b-knockdown cells spread slightly faster on FN and exhibited a striking phenotype characterized by the formation of multiple irregular protrusions (Figure 5). Knockdown cells were highly contractile and displayed increased retrograde movement of cell adhesions. Analysis of focal adhesion turnover revealed that control cells underwent normal focal adhesion turnover, with small adhesions present at the leading edge, and mature, stable adhesions located farther inside the cell (Figure 5B). STAT5b-knockdown cells readily formed adhesions at the leading edge of protrusive lamellipodia, but over time, these protrusions contracted, and the adhesions underwent retrograde flow back into the cell (Figure 5B). As a whole, these data suggest that STAT5b contributes to the coordinated regulation of protrusive and contractive processes.

Discussion

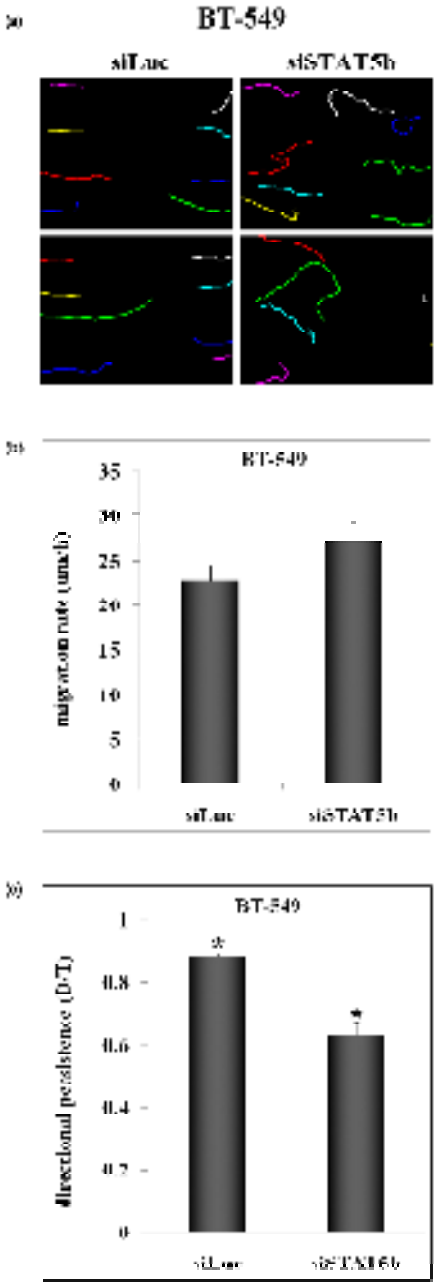
The work presented here establishes an important, previously undiscovered role for STAT5b in the migration of highly aggressive breast cancer cells. Knockdown of STAT5b inhibits migration of BT-549 and MDA-MB-231 human breast cancer cells to serum and fibronectin (Figures 1 and 3). This inhibition can be rescued by expression of wt-, Y699F-, or dn-STAT5b, but not with the SH2 domain mutant R618K-STAT5b (Figure 2). Upon attachment to fibronectin, STAT5b-

Figure 3



STAT5b knockdown inhibits β1-integrin-mediated migration to fibronectin (FN). **(a)** The undersides of trans-well filters were coated with 10 μg/ml FN or vitronectin (VN) overnight at 4°C. BT-549 and MDA-MB-231 cells were placed in serum-free media in upper chambers, and 1% fetal bovine serum (FBS) (BT-549) or 10% FBS (MDA-MB-231) medium was placed in the lower chambers for FBS controls. Serum-free medium was placed in lower chambers for FN and VN conditions. Migration was allowed to proceed for 3 hours (BT-549) or 6 hours (MDA-MB-231) (n = 3). (b) BT-549 and MDA-MB-231 cells were pretreated for 1 hour at 37°C with 10 μg/ml β_1-integrin-blocking antibody or DMSO control (con). Cells were plated in trans-well chambers in the presence of blocking antibody, and migration to 1% FBS (BT-549) or 10% FBS (MDA-MB-231) was measured. Student's *t* test was used to determine statistical significance ($P < 0.05$) between the following: BT-549: con and antibody (*) (n = 6); MDA-MB-231: con and antibody (*) (n = 4). **(c)** The undersides of trans-well filters were coated with 3 μg/ml FN overnight at 4°C, and migration assays were performed with siRNA-transfected cells as described in part a. One-way ANOVA with Tukey's post-test was used to determine statistical significance ($P < 0.05$) between the following: BT-549: siLuc and siSTAT5b FBS (*), siLuc and siSTAT5b FN (black circles); n = 4. MDA-MB-231: siLuc and siSTAT5b FBS (*), siLuc and siSTAT5b FN (black circles); n = 3.

Figure 4



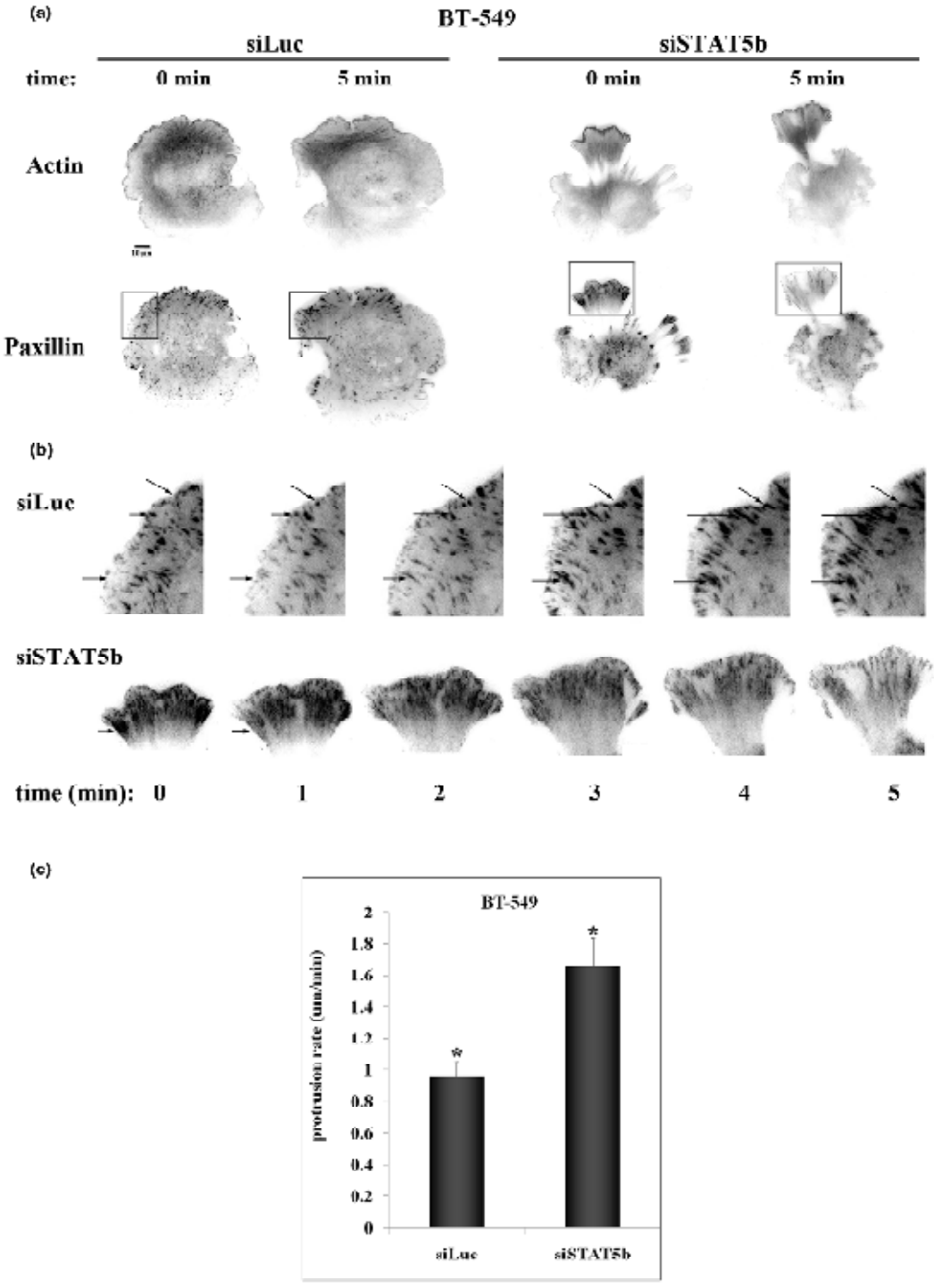
STAT5b knockdown negatively affects directionality during wound closure. Wounds were made in confluent monolayers of siRNA-transfected BT-549 cells, and time-lapse microscopy was used to track cell movement into the wound over a 6-hour period. **(a)** Progressive line images tracking single cell nuclei of cells along the wound edge. Two independent experiments are shown. **(b)** Migration rate (μm/h) was calculated by dividing the length of the migration path by the total movie time. **(c)** Directional persistence (D/T) was determined as the net displacement (D) divided by the total length of the migration path (T). (b, c) Values from at least four independent experiments were used for calculations (siLuc, n = 24; siSTAT5b, n = 32). Student's *t* test was used to determine statistical significance ($p < 0.05$) between siLuc and siSTAT5b (*). Comparisons of migration rate were not statistically significant.

knockdown cells form multiple, contractile protrusions resulting in loss of directionality and inefficient migration (Figures 4 and 5).

Knockdown of STAT5b inhibits migration of both BT-549 and MDA-MB-231 breast cancer cell lines to serum, whereas knockdown of STAT5a has no effect (Figure 1). These results may seem contradictory to those published by Sultan and colleagues [10], which reported a suppressive effect of STAT5a on breast cancer cell migration. However, in those studies, STAT5a was overexpressed in the moderately migratory BT-20 and T-47D breast cancer cell lines, which contain little to no endogenous STAT5a. The STAT5a and STAT5b expression pattern in those cell lines is very different from that seen in MDA-MB-231 cells, and consequently, the signaling may differ. Moreover, the role of endogenous STAT5b was not investigated in the studies of Sultan and associates. We and others found that STAT5b is the predominant STAT5a/b protein expressed in breast cancer cell lines and tissues, and that STAT5b, not STAT5a, mediates proliferation of breast cancer cells [10,14,16]. Differential expression or activity (or both) of STAT5a and STAT5b has been reported in other cancer model systems [9,24,25]. STAT5b levels and phosphorylation are elevated in SCCHN tumors compared with control mucosa, whereas STAT5a levels to do not change [24]. Consistent with these findings, antisense inhibition of STAT5b, but not STAT5a, inhibits *in vivo* growth of SCCHN xenografts [24]. In prostate cancer, differential STAT5a and STAT5b protein expression can be correlated with metastatic potential. STAT5a is expressed in nonmetastatic C1D mouse prostate cancer cells, but not in their metastatic C2H counterparts, whereas STAT5b is expressed in both [9]. Additionally, STAT5a is expressed in LNCaP human prostate cancer cells but not the more highly migratory PC-3 prostate cancer cell line, but STAT5b levels are comparable [9]. For these reasons, it is imperative to determine the individual contributions of STAT5a and STAT5b, as they may have distinct functions in tumorigenesis.

After establishing the necessity of STAT5b for maximal migration of breast cancer cells to serum, we investigated the mechanism by which this occurs. Interestingly, the migratory function of STAT5b does not require phosphorylation at tyrosine 699 (Y699) or the C-terminal transactivation domain (TAD), evidenced by equivalent rescue of migration with either wt-, Y699F-, or dn-STAT5b (Figure 2). Phosphorylation of Y699 is a hallmark of STAT5b transcriptional activation. If this residue is mutated to a phenylalanine, such that it cannot be phosphorylated (Y699F), the resulting STAT5b mutant is transcriptionally inactive [17,20]. Dn-STAT5b is also transcriptionally inactive because of the loss of the TAD and the ability to bind transcriptional cofactors [14]. Although STAT proteins are predominantly thought of as transcription factors that function in the nucleus, recent work identified a non-transcriptional, cytoplasmic role for unphosphorylated STAT3 in regulating

Figure 5



STAT5b-knockdown cells exhibit multiple protrusions with increased retraction. BT-549 cells were transfected with siRNA (siLuc or siSTAT5b), GFP-speckle-actin, and mKO-paxillin. Seventy-two hours after transfection, cells were plated on 3 μg/ml fibronection (FN) for 20 to 30 minutes, and TIRF time-lapse microscopy was performed. Images were taken every 3 seconds for 5 minutes at 60× magnification. (Line indicates 10 μm). **(a)** Still images representing actin and paxillin staining in control (siLuc) and STAT5b-knockdown (siSTAT5b) cells at start of filming (0 min) and end of filming (5 min). **(b)** Enlarged images depicting boxed areas from part a. Arrows identify single focal adhesions at each time point. **(c)** Protrusion rate (μm/min) was calculated as the length of the protrusion divided by the total time of the movie for at least three independent experiments (siLuc, n = 16; siSTAT5b, n = 13). Student's *t* test was used to determine statistical significance ($P < 0.05$) between siLuc and siSTAT5b (*).

tubulin dynamics [26]. Unphosphorylated STAT dimers take on an antiparallel configuration through interactions between the DNA-binding domains [27,28]. In this conformation, the SH2 domains are on opposite sides of the structure and are free to interact with phosphorylated tyrosines of other proteins. The necessity of the SH2 domain of STAT5b in migration is consistent with a cytoplasmic function of unphosphorylated STAT5b. We propose that STAT5b uses its SH2 domain to act as a scaffolding protein, bringing together signaling molecules necessary for efficient, directional migration. Although migration is inhibited to similar levels after STAT5b knockdown or blocking of β_1-integrin (Figure 3), it remains to be determined whether STAT5b interacts directly with β_1-integrin or if this effect is indirect. There are many tyrosine-phosphorylated proteins in the cytoplasm involved in migratory signaling with which STAT5b may interact, and future studies are aimed at uncovering these associations.

Loss of STAT5b leads to a polarity defect that impedes directional movement (Figure 4). During spreading on fibronectin, STAT5b-knockdown cells take on a remarkable phenotype distinguished by the formation of multiple, unstable protrusions (Figure 5). No defect is found in initial attachment, and once attached, protrusions extend rapidly. However, these protrusions are highly dynamic, and over time, they contract back into the cell. This phenotype is indicative of disrupted equilibrium between Rho family GTPases. Rac is localized predominantly at the leading edge, with Rho in the tail. Normal ratios of Rho and Rac lead to Rac-mediated formation and spreading of broad lamellipodia in the front of the cell, followed by Rho-mediated tail retraction, overall resulting in directional migration toward a stimulus [29]. Increased Rho activity in the front of the cell would disrupt this equilibrium and could account for the multiple protrusions and increased contraction at the front of the cell seen with STAT5b knockdown. Based on the data presented here, we postulate that unphosphorylated STAT5b mediates migration of breast cancer cells through regulation of cytoplasmic Rho GTPase family signaling.

In summary, these studies are the first to report a role for STAT5b in the migration of breast cancer cells. It is well established that STAT5b positively regulates breast cancer cell proliferation and survival, two processes important for initial tumor formation and growth. These data implicate STAT5b in the later stages of tumorigenesis also, such as migration. Future studies will further elucidate the mechanism by which STAT5b exerts its effect on migration, thereby broadening our understanding of how STAT5b promotes tumorigenesis and possibly metastasis. This will facilitate the long-term goal of defining conditions whereby STAT5b would be an effective therapeutic target for the treatment of breast cancer.

Conclusions

Our studies demonstrate a novel function of STAT5b in breast cancer cell migration. The importance of STAT5b in mediating breast cancer cell proliferation and survival has already been established. This work is the first to show a positive regulatory role for STAT5b in breast cancer cell migration. Therefore, STAT5b not only may function in the initiation of tumorigenesis, through its pro-proliferative and pro-survival signaling, but also may promote tumor progression by mediating migration.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TMB, CMS, and JTP designed the studies, and TMB performed the experiments. JZ assisted with the TIRF microscopy. All authors analyzed the data, and TMB drafted and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

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